

Photoaffinity Labelling of Botulinum Neurotoxin by Azido-ATP

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INTRODUCTION

Clostridium botulinum produces an exotoxin which is responsible for the severe and frequently fatal symptoms associated with botulism food poisoning (for reviews, see 1, 2). Thus far, seven serologically distinct botulinum neurotoxins (Botx) have been identified and termed A-G. Upon purification, each of the serotypes was found to have a number of similarities in its molecular properties. Their molecular weights, while not precisely the same, are about 150,000. Each of the serotypes has a protease-sensitive site, the cleavage of which produces a "nicked" form of the toxin. The nicked toxin is composed of two disulfide-linked fragments, one of about 100,000 mw (commonly termed the heavy chain) and another of approximately 50,000 mw (correspondingly, the light chain). Depending upon the serotype, endogenous nicking of toxin in culture can range from essentially zero to virtually complete; nicking appears to be required for the full expression of toxicity.

The pharmacology of Botx has been a subject of widespread interest for many years. Clearly, the toxin acts by blocking the release of acetylcholine from the presynaptic nerve terminals; no direct postsynaptic effects have been reported. On the cellular or molecular level, little definitive information is available. It appears that uptake of acetylcholine precursors, synthesis of acetylcholine, or packaging for release are not affected. Rather, it seems that some stage or component of the calcium-dependent exocytosis is rendered inactive (1,2).

While important details are not yet clear, there is suggestive evidence that Botx acts in the same general manner as other dichain bacterial toxins such as diphtheria toxin, cholera toxin, or pertussis toxin (3). Specifically, one chain (or oligomer) functions as a receptor recognition moiety and targets the toxin to a particular tissue or cell type, while the other chain is an enzyme which acts intracellularly. While there are some clues that the receptor for Botx may be, in part, a ganglioside, there have been no indications thus far as

to what the putative enzymatic activity of Botx might be. Because of the pivotal role played by nucleotides in the enzymatic activities of other bacterial toxins, we sought to determine whether Botx might also bind nucleotides. We employed the technique of photoaffinity labeling and identified several nucleotides or cofactors which bind to the heavy chain of the toxin. The results are reported here.

MATERIALS AND METHODS

Botulinum neurotoxins, serotypes B, C and E, were produced and purified at this Institute by published methods (4-6). The photoaffinity probe, 8-azido ATP, labeled in the gamma position with 32 P, was synthesized according to published procedures (7). Purified gangliosides were the generous gift of Dr. Oeri Alving, while other compounds were obtained from commercial sources. The labeling reaction was carried out by mixing toxin (10 µg), buffer (10 mM HES, 0.5M KCl, 2 mM MgCl_2 , pH 7), 32 P-azido-ATP and, when stipulated, competitor in a porcelain dish (final volume 100 µl). Activation of the reaction was achieved by a 30-sec exposure with a hand-held ultraviolet lamp (254 nm). An aliquot of each reaction mixture was removed and electrophoresed on a standard slab SDS PAGE system. The slabs were fixed with acetic acid, stained with coomassie blue, and extensively destained to remove the unincorporated photo-label. Following autoradiographic analysis, incorporation was quantitatively assessed by slicing the gel and assaying for radioisotope by Cherenkov counting.

RESULTS

Covalent association of radiolabeled azido-ATP with Botx was absolutely dependent on activation by light, as is demonstrated by the data in Fig. 1.

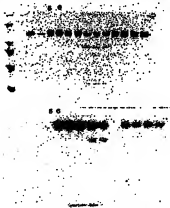


Fig. 1. Demonstration of UV light requirement for incorporation of azido-ATP into Botx.

The top frame, which shows the coomassie blue staining patterns, indicates no discernible effect of the UV light on the apparent molecular weight of the toxin (lane 6) vs the control of no UV activation (lane 5). An autoradiograph of the same gel (bottom frame) shows clearly that no appreciable radiolabel was covalently bound to the toxin in the absence of the activating UV light (lane 5). Thus, under our experimental conditions, botulinum toxin was not employing the azido-ATP in an autophosphorylation reaction.

In an attempt to determine whether the azido-ATP was binding to the heavy or light chains of the toxin, we carried out the photolabeling process and then treated the labeled toxin with trypsin to bring about a complete nicking. The sample was treated with reductant and electrophoresed as usual.

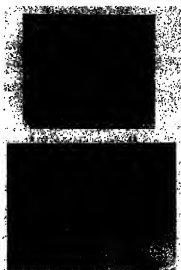


Fig. 2. Demonstration that azido-ATP is covalently incorporated into Botx heavy chain.

Examination of the coomassie blue-stained gel (Fig. 2) showed that trypsin treatment had completely converted the toxin (lane 3, top frame) to its constituent fragments (lane 12, top frame). Corresponding autoradiographs indicated that virtually all of the incorporated radiolabel was found on the heavy chain of the toxin (bottom frame). In experiments with different Botx serotypes, B, C, and E (data not shown), we obtained similar results and never observed appreciable incorporation of azido-ATP into the light chain of the toxin.

The binding of azido-ATP to Botx appeared to be saturable. Fig. 3 shows the results of two binding isotherm experiments.

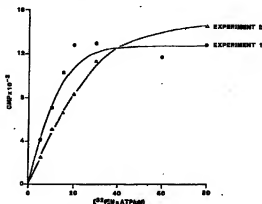


Fig. 3. Demonstration of saturation of amido-ATP-Robs binding.

As the concentration of photoaffinity label increased, the amount of incorporation also increased. However, a concentration was attained (c.e. 30 μ M) above which no additional incorporation was observed. The photolabel concentration yielding half-maximal incorporation was about 10 μ M.

An alternative approach to demonstrating saturability is to determine the level of radiolabeled amido-ATP incorporation in the presence of increasing concentrations of nonradioisotopic amido-ATP. When performed, these experiments, too, yielded results indicative of a saturable process. As shown by the data in Fig. 4, nonisotopic amido-ATP competes well with the radiolabeled compound.

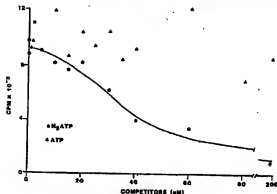


Fig. 4. Competition for radiolabeled azido-ATP binding to Botx by unlabeled azido-ATP and ATP.

The concentration required to block about 50% of the radiolabel incorporation was 20-30 μM , a value similar to the half-maximal concentration determined by experiments such as that in Fig. 3. At the highest concentrations of competitor tested, radiolabel incorporation was blocked 85-90%, indicating a non-specific binding of only 10-15%.

We also tested ATP as a competitor for azido-ATP in the concentration range found effective with nonradiolabeled azido-ATP (Fig. 4). Clearly, ATP does not assuressly block azido-ATP binding at a concentration where competition by the nonradioactive compound is complete. Indeed, it required millimolar ATP levels to observe any inhibition of azido-ATP-Botx binding. This was also true for other common triphosphate nucleotides such as CTP, UTP, ITP, TTP, and GTP (data not shown).

In an attempt to determine what the natural ligand for the azido-ATP binding site on Botx might be, we tested a wide array of nucleotides, cofactors, etc., for their potential as competitors of the interaction (selected data presented in Table 1).

TABLE 1
COMPETITION FOR ASIDO-ATP LABELING BY COFACTORS,
GANGLIOSIDES, AND OTHER COMPOUNDS

| | |
|------------------------------|-------------|
| NADH | 10 μ M |
| NADPH | 10 μ M |
| NAD ⁺ | 1 mM |
| NADP ⁺ | 1 mM |
| NIACIN | 1 mM |
| NICOTINAMIDE | 1 mM |
| ADP-RIBOSE | 100 μ M |
| THIOURELLINE | 100 μ M |
| FMN | 10 μ M |
| FOLIC ACID | 10 μ M |
| G _{M1} GANGLIOSIDE | 15 μ M |
| GD _{1A} GANGLIOSIDE | 10 μ M |
| GD _{1B} GANGLIOSIDE | 15 μ M |
| G _{M13} GANGLIOSIDE | 10 μ M |
| G _Q GANGLIOSIDE | 5 μ M |
| N-ACETYLHOMANINIC ACID | 1 mM |

Competition was assessed by including the indicated compound in the standard protocol and comparing the degree of labeling with a control. Value given is the concentration which inhibits radiolabel binding by 50%.

None of the purine or pyrimidine nucleotides we tried inhibited the labeling at concentrations below 1 mM. We did find several cofactors which did compete for the labeling of Biotin by asido-ATP. NADH and NADPH both blocked the binding at 10 μ M concentrations, while NAD⁺ and NADP⁺ had no effect up to 1 mM. Two other cofactors which competed at micromolar concentrations were folic acid and flavin mononucleotide (FMN). Since molecules such as NADH or FMN are good reducing agents, we considered the possibility that they were directly inactivating the asido-ATP by an oxidation-reduction reaction. This was tested spectrophotometrically with negative results.

The other class of molecules which were very effective at competing for the photolabeling reaction were gangliosides. Table 1 lists the specific gangliosides we tested and the lowest concentrations which gave a visually-estimated 50% desaturation. There was a degree of specificity in the competition by various gangliosides, roughly paralleling the previously reported (10) inhibition of Biotin toxicity by these compounds. That is, the complex gangliosides G_{M13} and G_Q were more effective at competing for asido-ATP labeling of Biotin than the simpler gangliosides such as G_{M1}. The differences in potencies were not nearly as pronounced as were observed in the toxicity tests, but were specific in the sense that steric acid alone was a very poor competitor.

DISCUSSION

Nucleotides are known to be important in the mechanism of action of several bacterial toxins. This study was undertaken in an attempt to determine whether nucleotides might play a role in the toxicity of Botx. We chose to use azido-ATP for our work because of its high reactivity and ability to interact with a range of nucleotide sites (8,9). We found azido-ATP bound to a site on the heavy chain of Botx serotypes B, C, and E. In preliminary experiments, we also observed azido-ATP binding to tetanus toxin heavy chain (data not shown). The binding to Botx was specific in that it was saturable, could be blocked by excess, unlabeled azido-ATP, and had an apparent dissociation constant of around 10 μ M. However, as judged by the lack of competition by a wide variety of nucleotides (including ATP itself), the binding site recognised by azido-ATP has some curious properties. For example, although none of the common mono-, di-, or triphosphate nucleotides tested were especially potent at competing for azido-ATP binding (data not shown), it was still apparent that a nucleoside was a better competitor than a nucleotide and that potency was inversely related to the number of phosphates on the latter. Another interesting feature of competition was that MAD^+ and MADP^+ were barely effective in the millimolar range, while their reduced forms (MADH^+ and MADPH^+) competed well at 10 μ M. This is exactly the opposite specificity seen with the MAD^+ site on diphtheria toxin (11) and suggests that the azido-ATP site on Botx is different. The facts that gangliosides are probably a component of the receptor for Botx and that the receptor-recognition site is apparently on the heavy chain are consistent with our work. We found gangliosides competed for the binding of azido-ATP to Botx and could show that the photo-label was incorporated into the heavy chain. It may be that the azido-ATP binding site on Botx is more analogous to the regulatory "P site" (12) on diphtheria toxin than to a (putative) enzymatic site. However, either comparison is speculative on our part and further work is needed to define the biological relevance of the binding reported here.

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DEVELOPMENT OF RECOMBINANT VACCINES FOR BOTULINUM NEUROTOXIN

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L. A. Smith. Development of recombinant vaccines for botulinum neurotoxin. *Toxicon* 36, 1539-1548, 1998.—Synthetic genes encoding non-toxic, carboxyl-terminal regions (~50 kDa) of botulinum neurotoxin (BoNT) serotypes A and B (referred to as fragment C or H_C) were constructed and cloned into the methylotrophic yeast, *Pichia pastoris*. Genes specifying BoNTA(H_C) and BoNTB(H_C) were expressed as both intracellular and secreted products. Recombinants, expressed intracellularly, yielded products with the expected molecular weight as judged by SDS-PAGE and Western blot (immunoblot) analysis, while secreted products were larger due to glycosylation. Gene products were used to vaccinate mice and evaluated for their ability to elicit protective antibody titers *in vivo*. Mice given three intramuscular vaccinations with yeast supernatant containing glycosylated BoNTA(H_C) were protected against an intraperitoneal challenge of 10⁶ 50% mouse lethal doses (MLD₅₀) of serotype A neurotoxin, a result not duplicated by its BoNTB(H_C) counterpart. Vaccinating mice with cytoplasmically produced BoNTA(H_C) and BoNTB(H_C) protected animals from a challenge of 10⁶ MLD₅₀ of serotype A and B toxins, respectively. Because of the glycosylation encountered with secreted BoNT(H_C), our efforts focused on the production and purification of products from intracellular expression. Published by Elsevier Science Ltd

INTRODUCTION

Botulism is a severe neuroparalytic disease caused by one of seven neurotoxins produced by the bacterium, *Clostridium botulinum*. With a lethal dose in the range of 10⁻⁹ g per kg body weight (Lamanna, 1959), botulinum neurotoxins are the most lethal agents nature has produced. The neurotoxins function by binding to specific receptors on peripheral cholinergic nerve cells (Black and Dolly, 1986a). They enter the cells by an internalization process (Black and Dolly, 1986b), and once translocated inside the cells, prevent the evoked release of acetylcholine by inactivating neuronal proteins associated with the neuroexocytosis apparatus (reviewed by Montecucco and Schiavo,

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† The views of the author do not purport to reflect the positions of the Department of the Army or the Department of Defense (para. 4-3, AR 360-5).

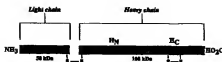


Fig. 1. Schematic structure of botulinum neurotoxins. BoNT is synthesized as a single polypeptide chain which can be cleaved at a protease-sensitive site resulting in a light and heavy chain bound by a disulfide bond. The heavy chain is divisible functionally into an amino-terminal domain (H_{1N}) and a carboxyl-terminal domain (H_{1C}).

1994; Montecucco *et al.*, 1994; Lebeda and Singh, submitted). Clinical manifestations of the disease are dominated by the neurological signs and symptoms resulting from a toxin-induced blockade of the voluntary motor and autonomic cholinergic junctions. Symmetrical cranial nerve impairment affecting the bulbar musculature frequently mark the onset of symptoms such as ptosis, ambliopia and/or blurred vision, dysphonia, and a dry, sore throat. Flaccid paralysis of the pharyngeal and laryngeal muscles give rise to dysphagia and flaccid dysarthria. If the botulism is a consequence of ingesting improperly preserved foodstuffs contaminated with bacteria and their preformed toxins, nausea, abdominal pain, vomiting, and diarrhea may often precede or accompany the neurologic indications. As the disease progresses, a descending paralysis ensues in which the neck muscles, respiratory muscles, and muscles in the extremities and trunk are affected. The paralysis of the respiratory muscles leading to dyspnea or ventilatory failure is the cause of death in a botulinum intoxication.

The sporulating, obligate anaerobic, gram-positive bacillus *C. botulinum* produces seven forms of antigenically distinct exotoxins which are differentiated serologically by specific neutralization (Hatheway, 1992). They have been designated as serotypes A, B, C₁, D, E, F and G. Polyclonal antibodies derived for a specific neurotoxin can neutralize the toxic effects of that toxin but will not cross-neutralize another toxin serotype. Structurally, the toxins are produced as single-chain polypeptides having molecular masses (M_r) of approximately 150 kDa. Most of the clostridial strains contain specific endogenous proteases which activate the toxins at a protease-sensitive loop (DasGupta, 1989) located approximately one-third of the way into the molecule from the amino-terminal end. The proteolytic nicking generates two fragments which are held together by a single disulfide bond (Fig. 1). Upon reduction and fractionation (electrophoretically or chromatographically), the two chains can be separated; one chain has a M_r of ~100 kDa and is referred to as the heavy chain while the other has a M_r of ~50 kDa and is termed the light chain.

Botulinum neurotoxin (BoNT) has three functional domains, a receptor-binding domain, a translocation domain, and an enzymatic domain (reviewed by Montecucco and Schiavo, 1994; Montecucco *et al.*, 1994; Halpern and Neale, 1995). Part of the receptor-binding domain resides towards the carboxyl-terminal portion of the toxin, a region known as the fragment C or H_{1C} region. The amino-terminal region of the heavy chain (H_{1N}) and a small portion of the light chain spanning the disulfide bridge appears to be involved in the translocation or internalization of the light chain into the cell (Zhou *et al.*, 1995). The light chain(s) are zinc-dependent endoproteases which selectively inactivate three essential proteins involved in the docking and fusion of acetylcholine-containing synaptic vesicles to the plasma membrane. The light chains of BoNT serotypes A, C₁, and E cleave SNAP-25 (synaptosomal-associated protein of M 25,000), serotypes B, D, F and G cleave VAMP/synaptobrevin (synaptic vesicle-

associated membrane protein); and serotype C₁ cleaves syntaxin. Inactivation of SNAP-25, VAMP, or syntaxin by BoNT leads to an inability of the nerve cells to release acetylcholine resulting in neuromuscular paralysis and possible death, if the condition remains untreated.

RESULTS AND DISCUSSION

In the development of a recombinant vaccine to protect against BoNT and the effects caused by the toxin, we demonstrated a number of years ago that we could subclone segments of the BoNTA gene and express these non-toxic fragments in *Escherichia coli* (Zuber and Smith, unpublished results). We were then able to vaccinate mice with the non-toxic recombinants and test their ability to elicit protective immunity *in vivo*. Clones pCBA2, pCBA3, and pCBA4 (gift from Nigel Minton) containing overlapping gene fragments of the BoNTA gene (Thompson *et al.*, 1990), were the source for templates used to produce the various antigens to be examined. We prepared smaller versions of the gene fragments in pCBA2, pCBA3, and pCBA4 by cutting with restriction enzyme(s) and subcloning into vectors such as the pWS50 (Fig. 2). We also amplified by PCR, specific gene segments and subcloned them into expression plasmids such as pET vectors. Expressed products were identified by SDS-PAGE and Western blot analysis, partially purified, and used to vaccinate mice. The mice were then challenged with active neurotoxin after vaccination. Antigens representing various regions from the three functional domains of the toxin (Fig. 3) were analyzed for their ability to elicit protective antibodies in mice. Of all the fragments we, and others (LaPenotiere *et al.*, 1995; Dertzbaugh and West, 1996) tested, only one was able to significantly protect mice (Table 1). This was the fragment located at the carboxy-terminus of the toxin (~50 kDa) designated as the fragment C region. Our subsequent efforts to develop vaccine candidates to protect against BoNT forthwith focused exclusively on the H_C region.

Recent advances in biotechnology make available various options for expressing and producing protein products. Having shown that a non-toxic fragment of BoNT could induce a formidable protective immunity in animals, our efforts were now directed towards selecting and integrating an expression system into our vaccine program which would produce the maximum amount of properly folded, immunogenic antigen.

Table 1. Survival of vaccinated mice challenged with BoNT

| Immunogen | (Alive/total) | | |
|------------------------|------------------------|----------------------|-----------------------|
| | 10 MLD ₅₀ * | 50 MLD ₅₀ | 100 MLD ₅₀ |
| BT702 | 0/10 | — | — |
| BT913 | 0/10 | — | — |
| BT1025 | 0/10 | — | — |
| BT820 | 0/10 | — | — |
| BT1143 | 0/10 | — | — |
| BT1145 | 0/10 | — | — |
| BT1143 and BT1146 | 10/10 | 0/10 | — |
| BT1150 | — | — | 0/10 |
| BT1151 | — | — | 0/10 |
| BT(Hc) | 10/10 | — | 10/10 |
| <i>E. coli</i> extract | 0/10 | — | — |
| BoNT-A toxoid | 10/10 | — | 10/10 |

*MLD₅₀ (50% mouse lethal dose) is an empirically-determined dose of neurotoxin necessary to cause lethality in 50% of a population of mice.

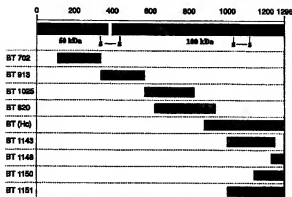


Fig. 3. Diagrammatic presentation of regions of BoNTA expressed in *E. coli* and subsequently tested for their ability to elicit neutralizing antibodies *in vivo*.

Because of the high-level of expression, many laboratories (Cregg *et al.*, 1987; Wegner, 1990; Buckholz and Gleeson, 1991) reported the expression of their heterologous genes in the yeast expression system, *Pichia pastoris*. The tetanus neurotoxin fragment C (TeNT(H_C)) reportedly produced yields of 12 g/L (Clare *et al.*, 1991) when expressed in *P. pastoris*. We investigated the *P. pastoris* system for the expression of BoNT(H_C) genes. Previously, investigators working on the expression of TeNT(H_C) reported difficulties in obtaining optimal expression levels when expressing the natural gene of TeNT(H_C) in *E. coli* (Makoff *et al.*, 1989) and in yeast (Romanos *et al.*, 1991). Makoff *et al.* showed that the low levels of expression in *E. coli* were due to the presence of rare codons in the natural gene of *C. tetani*. Substituting the rare codons with those more frequently found in the *E. coli* host eliminated impediments to high-level expression. Expression of the natural gene of TeNT(H_C) in yeast was even more troublesome and appearance of full-length product was never observed. In a very elegant experiment, Romanos *et al.* (1991) showed that the high adenine and thymine (A + T) rich base composition found in the natural gene of clostridial DNA gave rise to fortuitous transcriptional termination signals in yeast. However, this obstacle could be overcome by preparing a synthetic gene in which codon usage was altered as to greatly reduce the A + T content (Clare *et al.*, 1991; Romanos *et al.*, 1991; Romanos *et al.*, 1992). We prepared synthetic genes for the H_C of BoNT in which we decreased the A + T base content, reducing the possibility of incidental, unforgiving transcriptional termination signals in the BoNT(H_C) genes.

The first synthetic gene constructed encoded the BoNTA (H_C). It was initially expressed in *E. coli* and elicited significant protective immunity *in vivo* (Clayton *et al.*, 1995). The synthetic gene encoding the BoNTA(H_C) was modified for insertion into the secretory vector pPIC9K (gift from Phillips Petroleum, Bartlesville, OK) and the construct was integrated into the chromosomal *AOX1* locus of *P. pastoris* strain GS115 (Smith, unpublished results). Clones were selected based on their ability to express histidine dehydrogenase (Cregg *et al.*, 1993) and aminoglycoside phosphotransferase 3'

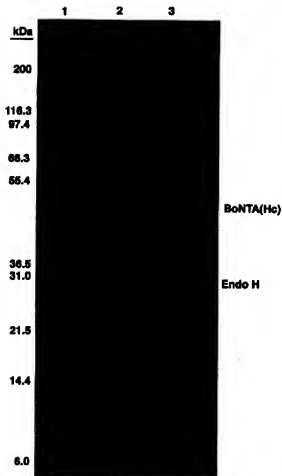


Fig. 4. SDS-PAGE of secreted recombinant BoNTA(Hc) from *Pichia pastoris*. Lanes: 1, Novex (San Diego, CA) Mark 12[®] protein standard; 2, aliquot of fermentation broth containing secreted BoNTA(Hc); 3, aliquot of fermentation broth treated with Endo H.

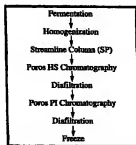
Table 2. Survival of challenged mice vaccinated with recombinant BoNTA(H_C) produced by *Pichia pastoris*

| Mice | BoNTA challenge | |
|----------|---|---|
| | 10 ⁵ MLD ₅₀ (alive/total) | 10 ⁶ MLD ₅₀ (alive/total) |
| Group I | 10/10 | 10/10 |
| Group II | 10/10 | 10/10 |
| Controls | 0/10 | 0/10 |

Group I received 15 µg and group II received 30 µg of total secreted protein at 0, 2 and 4 weeks. Controls were naive mice.

(1) (Schorer *et al.*, 1994) markers. The clones were subsequently analyzed for their ability to express and efficiently secrete the BoNTA(H_C) product into the medium. An aliquot of recombinant product was analyzed on SDS-PAGE (Fig. 4, lane 2). The four multiple bands on the SDS-PAGE (Fig. 4, lane 2) observed migrating below the 55.4 kDa protein marker (Fig. 4, lane 1), as well as the higher *M_r* bands visualized above the 66.3 and 116.3 kDa markers (Fig. 4, lane 1), were revealed by Western blot analysis (data not shown) and treatment with Endo H (Fig. 4, lane 3) to be glycosylated BoNTA(H_C). Secreted BoNTA(H_C) was capable of eliciting a protective immunity in mice (Table 2). Mice immunized with three doses of 15 µg of total secreted protein were asymptomatic to a challenge of 10⁶ MLD₅₀ of BoNTA while non-immunized mice succumbed within a few hours. However, glycosylated BoNTB(H_C) (Smith, unpublished results) and glycosylated TeNT(H_C) (Romanos *et al.*, 1991) failed to elicit protective immunity in mice, although deglycosylation restored its capacity to protect animals. Core- and to a minor extent hyper-glycosylation of the BoNT(H_C) results as the protein traverses the secretory pathway of the yeast cell, becoming glycosylated in the endoplasmic reticulum, and then further processed in the Golgi cisternae before being secreted from the cell.

Conceptually, three courses of action could be taken to generate a non-glycosylated rBoNT(H_C) protein product: (1) a deglycosylation step could be incorporated into the manufacturing process during production to remove carbohydrate moieties, (2) the potential glycosylation site(s) (Kornfeld and Kornfeld, 1985) in the protein could be altered by protein engineering techniques, or (3) the product could be expressed intracellularly avoiding the secretory pathway altogether. Increasing the complexity of an industrial scale manufacturing process by adding a deglycosylation step is not a

Fig. 5. BoNTB(H_C) production and purification process.

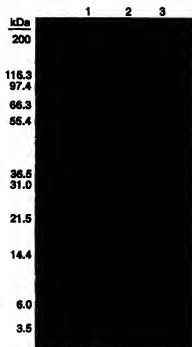


Fig. 6. SDS-PAGE of purified BoNTB(H_C) intracellularly expressed in *Pichia pastoris*. Lanes: 1, Novex (San Diego, CA) Mark 12[®] protein standard; 2, 4 μ g of BoNTB(H_C); 3, 8 μ g of BoNTB(H_C).

recommended alternative. Additionally, as the putative BoNTA(H_C) has nine potential N-linked glycosylation sites, BoNTB(H_C) has one, BoNTC(H_C) has eight, BoNTD(H_C) has three, BoNTE(H_C) has six, BoNTF(H_C) has six, and BoNTG(H_C) has ten, eliminating these sites by mutagenesis is not a practical alternative. Thus, we proceeded to express the BoNT(H_C) products intracellularly utilizing the yeast expression vector pHILD4 (gift from Phillips Petroleum, Bartlesville, OK).

The first synthetic gene we expressed intracellularly in *P. pastoris* encoded a putative H_C region for BoNT serotype B. The pHILD4 vector harboring the BoNTB(H_C) gene was integrated into the chromosomal *AOX1* locus of *P. pastoris* and isolated clones were analyzed for their ability to express rBoNTB(H_C) by SDS-PAGE and Western blot analysis (data not shown). After crude yeast extracts containing rBoNTB(H_C) were shown to protect mice (data not shown), one clone was selected for growth and methanol-induction in a fermentor. Yeast cells from the fermentation were lysed by

Table 3. Survival of challenged mice vaccinated with BoNTB(Hc) from *Pichia pastoris*

| Dosage* (μ g) | Two vaccinations, No. of survivors/total no. | | Three vaccinations at challenged dose of ^b : | |
|-----------------------|---|--------|--|--------|
| | 10^3 | 10^6 | 10^3 | 10^6 |
| 0.1 | | 19/20 | | 15/20 |
| 0.5 | | 20/20 | | 20/20 |
| 1.0 | | 20/20 | | 20/20 |
| 2.0 | | 20/20 | | 19/20 |
| Controls ^c | 0/10 | 0/10 | 0/10 | 0/10 |

*Immunogens were absorbed to Alhydrogel, administered intramuscularly at 2 week intervals and challenged 2 weeks after vaccination.

^bNeutralization results at 96 h.

^cControl mice were immunized with a yeast extract obtained from a clone in which the vector minus the BoNTB(Hc) gene had been integrated into the yeast chromosome and subsequently induced with methanol during fermentation.

using a Gaulin homogenizer and the rBoNTB(Hc) was purified by using cationic and anionic exchange chromatography (Fig. 5). The production and purification process developed for the rBoNTB(Hc) generated a highly purified product (Fig. 6) with a yield of 2.34 g of rBoNTB(Hc) per 6 kg wet yeast cell mass (30 L fermentation) (Potter *et al.*, submitted).

Akin to the rBoNTA(Hc) vaccine, rBoNTB(Hc) was also a remarkably efficacious immunogen (Table 3). Mice vaccinated with low doses (from 0.1 to 2.0 μ g) of the purified rBoNTB(Hc) vaccine candidate and subsequently challenged with 10^6 MLD₅₀ of active BoNTB exhibited a high survival rate (Table 3). Out of 160 mice vaccinated, 156 mice were asymptomatic to the challenge while all of the control animals expired whether they were challenged with 10^3 or 10^6 BoNTB MLD₅₀. Long-term immunity studies were performed using the recombinant BoNTA(Hc) and the BoNTB(Hc) vaccines. Mice vaccinated with three doses of vaccine remain protected against a challenge of 10^6 MLD₅₀ 12 months post-immunization (data not shown).

The first pilot lot of the rBoNTB(Hc) vaccine has been manufactured in compliance with U.S. Food and Drug Administration's current Good Manufacturing Practices (cGMP). The product is presently in preclinical trials being evaluated under current Good Laboratory Practices (cGLP) for safety, efficacy, and product stability. The BoNTA(Hc) vaccine candidate has been scheduled for pilot lot production and preclinical testing. The remaining synthetic genes encoding putative Hc for C₁, D, E, F, and G serotypes have been completed. Synthesis, cloning, and expression of the remaining genes encoding putative Hc for C₁, D, E, F, and G serotypes has been completed as part of the process for development of vaccine candidates for those serotypes of BoNT.

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Expression, purification, and characterization of *Clostridium botulinum* type B light chain

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Abstract

A full-length synthetic gene encoding the light chain of botulinum neurotoxin serotype B, approximately 50 kDa (BoNT/B LC), has been cloned into a bacterial expression vector pET24a+. BoNT/B LC was expressed in *Escherichia coli* BL21-DE3 pLysS and isolated from the soluble fraction. The resultant protein was purified to homogeneity by cation chromatography and was determined to be >98% pure as assessed by SDS–polyacrylamide gel stained with SilverXpress and analyzed by densitometry. Mass spectroscopic analysis indicated the protein to be 50.8 kDa, which equaled the theoretically expected mass. N-terminal sequencing of the purified protein showed the sequence corresponded to the known reported sequence. The recombinant BoNT/B light chain was found to be highly stable, catalytically active, and has been used to prepare antisera that neutralize against BoNT/B challenge. Characterization of the protein including pH, temperature, and the stability of the protein in the presence or absence of zinc is described within. The influence of pH differences, buffer, and added zinc on secondary and tertiary structure of BoNT/B light chain was analyzed by circular dichroism and tryptophan fluorescence measurements. Optimal conditions for obtaining maximum metalloprotease activity and stabilizing the protein for long term storage were determined. We further analyzed the thermal denaturation of BoNT/B LC as a function of temperature to probe the pH and added zinc effects on light chain stability. The synthetic BoNT/B LC has been found to be highly active on its substrate (vesicle associated membrane protein-2) and, therefore, can serve as a useful reagent for BoNT/B research.

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Botulinum neurotoxin, the causative agent of botulism poses a significant bioweapon threat because of its extreme lethality. An outbreak of botulism caused by dispersion of toxin is a serious public health emergency that requires an immediate response by the administration of botulinum antitoxin and often mechanical ventilation. Botulism is a neuroparalytic disease caused by seven immunologically distinct neurotoxins (types A–G) produced by *Clostridium botulinum*, a gram-positive, rod shaped, motile, non-encapsulated, spore-forming anaerobic bacterium. Botulinum

neurotoxin(s) (BoNT)¹ are expressed as single polypeptide chains with approximate molecular masses of 150 kDa. Most of the *C. botulinum* strains have endogenous proteases that nick the toxin at a protease-sensitive site, activating the toxin and generating a light chain and a heavy chain held together by a single disulfide bond. The light chain can be separated from the heavy chain by reduction followed by electrophoresis or chromatography. The smaller (50 kDa) N-terminal fragment is designated as the light chain (LC) while the C-terminal fragment (100 kDa)

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¹ Abbreviations used: BoNT, botulinum neurotoxin; LC, light chain; HC heavy chain; ECL, electrochemoluminescence; IPTG, isopropyl-β-D-thiogalactopyranoside; TB terrific broth; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; ELISA, enzyme-linked immunosorbent assay; BCA, bicinchoninic acid; cIEF, capillary isoelectric focusing; CD, circular dichroism; PBS, phosphate-buffered saline.

is referred to as the heavy chain (HC) [1]. The zinc-endopeptidase catalytic domain of the toxin resides in the 50 kDa N-terminal portion of the active protein.

After binding to peripheral cholinergic nerve cells, the toxin is internalized into endosomes through receptor-mediated endocytosis [2,3]. The amino terminal half of the HC is believed to participate in the translocation mechanism of the LC across the endosomal membrane [4–6]. Upon internalization into vesicular compartments, the catalytic LC is translocated to the cytosol where the final event of intoxication involves the catalytic hydrolysis of key synaptic vesicle proteins [7–9] by the light chain [10,11]. The zinc-dependent endoproteolytic LC selectively inactivates three essential proteins referred to as SNARE (soluble *N*-ethylmaleimide sensitive factor attachment protein receptors) proteins which are involved in the docking and fusion of acetylcholine-containing vesicles to the plasma membrane [12,13]. Botulinum neurotoxins (BoNT) A–G each have the capacity to cleave a protein of the neuroexocytosis apparatus: SNAP-25 is cleaved by BoNT/A [14], BoNT/C, and BoNT/E [15,16]. Serotype C also cleaves syntaxin [17,18]. The LC of BoNT/B cleaves VAMP/synaptobrevin [11]; serotype D, G, and F are also specific for VAMP/synaptobrevin [19–21]. Inactivation of SNAP-25, syntaxin, or VAMP by BoNT leads to an inability of the nerve cells to release acetylcholine, resulting in neuromuscular paralysis [22]. The LC by itself is nontoxic and does not translocate through the cell membrane of cholinergic cells.

Our laboratory has produced BoNT/B LC as a reagent to be used in high-throughput assays to screen for potential LC antagonists, to further elucidate the toxin's mechanism of action, and to study the immunological response to the catalytic domain of the toxin. The LC was cloned into an *Escherichia coli* expression system, pET24+, and the recombinant plasmid was transformed into BL21.DE3.pLysS cells. The LC was purified by successive cation-exchange chromatographic steps and characterized for purity, structural integrity, and enzymatic activity. Details of the production process and features of the recombinant BoNT/B LC are described within this study.

Materials and methods

Materials

All buffer reagents and components were from Sigma (St. Louis, MO) unless otherwise specified. Precast tricine gels, load buffer, running buffer, stains, molecular markers and oligonucleotides for the PCR reaction were obtained from Invitrogen (Carlsbad, CA). Plasmid pBlueScriptII was purchased from Stratagene (LaJolla, CA). Plasmid pET24+ was purchased from Invitrogen (Carlsbad, CA). The *E. coli* strain DH5α used in the cloning procedure and *E. coli* strain BL21.DE3.pLysS used for protein expression were purchased from Novagen (Madison, WI).

Electrochemiluminescence (ECL) reagents were purchased from Perkin Elmer Life Sciences (Boston, MA). Restriction endonucleases were obtained from New England Biolabs (Beverly, MA) and used according to the manufacturer's directions. The chromatography columns and resins used for purification were from Applied Biosystems (Foster, CA) and Pharmacia (Uppsala, Sweden). Anti-botulinum serotype B-specific polyclonal equine antibodies were used to verify protein expression. The equine polyclonal antibodies were obtained from PerImmune (Rockville, MD) for the Department of Defense. Isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Novagen (Madison, WI). The synthetic substrate peptide for type B LC contains residues 60–94 of human VAMP-2: acetyl-LSELEDDRADALQAGASFETSAALKLRKYWWKNLK-carboxamide was custom synthesized by SynPep (Dublin, CA).

Construction of the synthetic gene BoNT/B LC

Oligonucleotides were designed using the published sequence for Okra *C. botulinum* structural gene encoding the type B neurotoxin and were used in a series of ligation/PCRs to generate a final 1323-base pair (bp) fragment that was cloned into pBlueScript II at *Xho*I–*Xba*I sites. This enabled the synthesis of single-stranded DNA that was used for DNA sequencing. Verification of the resultant clone was then confirmed by DNA sequencing. Plasmid DNA used for cloning and single-stranded sequencing was prepared by using a kit purchased from Qiagen (Valencia, CA). The sequence was optimized for GC content and codon usage. Approximately 21% of the published sequence was altered by codon optimization. Oligonucleotides were designed that contained the restriction sites for *Nde*I and *Bam*HI to facilitate the cloning process. The full-length gene was excised from vector pBlueScript II and subcloned into *Nde*I and *Bam*HI digested pET24+ vector. The insert was ligated into pET24+ so as to begin expression with the initial methionine of the LC. Sequencing of the complete clone was performed and a single mutation was noted. In vitro mutagenesis was performed to correct the misincorporation, the correction was verified by sequence analysis. The resulting construct was used to transform, by calcium phosphate precipitation, *E. coli* BL21.DE3.pLysS cells for protein expression. Clones were assayed by Western blot for their ability to express BoNT/B LC.

Expression and purification of the LC

The bacteria was cultured in 1-L flasks containing terrific broth (TB) supplemented with 60 μ g/ml of kanamycin with vigorous shaking (220 rpm) at 37 °C until the cultures reached an OD₆₀₀ of 0.6. The recombinant BoNT/B LC protein was induced by adding IPTG (final concentration 0.1 mM) for 18 h. Cells were harvested by centrifugation and the pellet was immediately used or

stored frozen at -20°C . One gram of LC cell paste was resuspended in 20 ml of buffer (20 mM Tris, 2 mM EDTA, pH 5.0). The suspended cells were sonicated using an alternating cycle of sonication (30 s) and ice incubation (30 s) over a period of 12 min. To remove debris and insoluble material the supernatant was centrifuged 15 min, 4°C , 15 K. Additional buffer, 20 mM Tris, 2 mM EDTA, pH 5.8, was added to a final volume of 40 ml. The supernatant was sterilized with $0.2\text{ }\mu\text{m}$ filters. Following the filtering process the soluble portion was further purified using cation exchange chromatography and a BioCAD Model 700E (Perceptive Biosystems, Farmingham, MA).

A Poros HS 20 column was equilibrated with buffer (20 mM Tris, 2 mM EDTA, pH 5.8) before loading the protein. Protein was eluted from the column with a linear gradient of 20 mM Tris, 2 mM EDTA, 1 M NaCl, pH 5.8, 0–100% over 30 min at a rate of 1 ml/min. Throughout the gradient 1 ml fractions were collected. The peak eluted over several fractions which were collected and pooled. The column was washed extensively and was equilibrated with buffer (20 mM Tris, 2 mM EDTA, pH 5.4). The pooled fractions of run 1 were equilibrated to the new buffer. The protein was loaded on the Poros HS 20 column to further purify. The pooled fractions of run 2 were collected. A Source 15 S column was equilibrated with 20 mM Tris, 2 mM EDTA, pH 5.4, and the pooled fractions from run 2 were loaded through the buffer port. A linear gradient of 20 mM Tris, 2 mM EDTA, 1 M NaCl, pH 5.4, 0–100% over 30 min at a rate of 1 ml/min was performed. The peak fractions of run 3 were collected, pooled, and assayed for homogeneity. The expressed and purified recombinant BoNT/B LC was stored at -70°C in 20 mM Na acetate buffer with a pH of 5.8 containing 2 mM EDTA. Recovery of the BoNT/B LC was calculated to be 4 mg/g of cell paste.

Protein assays and SDS-PAGE

Total protein concentrations were determined by using bovine serum albumin as a protein standard and Pierce BCA (bicinchoninic acid) protein assay using the micro-scale protocol as the manufacturer directed. Electrophoresis was performed according to Laemmli [23] on a 10% acrylamide gel under reducing conditions with a Novex Mini-cell II apparatus (Novex, San Diego, CA); pre-stained SeeBlue markers were used to determine the size of the recombinant protein. Gels were stained and proteins were visualized using Coomassie brilliant blue R-250 and SilverXpress. Protein samples were further analyzed by Western blot analysis to confirm protein identity [24]. Proteins separated by SDS-polyacrylamide gels were transferred onto nitrocellulose followed by incubation with equine polyclonal antibody to BoNT/B toxin. Membranes were washed and then incubated with affinity purified goat anti-horse IgG (H + L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD) horseradish peroxidase antibody.

Detection was accomplished by using an enhanced chemiluminescence system (ECL).

N-terminal sequence and mass spectroscopic analysis of rBoNT/B LC

Following SDS-PAGE electrophoresis of the purified LC, the resolved protein was transferred onto a PVDF membrane. The appropriate PVDF band was sequenced using Edman degradation in an Applied Biosystems Procise Sequencer in the 0–20-pmol range. Molecular mass was determined by Matrix-assisted laser desorption/ionization (MALDI)-TOF Analysis. Trypsically digested BoNT/B LC was co-crystallized with α -cyano-4-hydroxycinnamic acid (Agilent Technologies, Palo Alto, CA) and spotted directly on a stainless steel matrix-assisted laser desorption ionization (MALDI) plate. Mass spectra were acquired using an Applied Biosystems 4700 MALDI-TOF/TOF mass spectrometer (Applied Biosystems, Foster City, CA). For all mass spectra the laser frequency was 200 Hz. MALDI spectra were internally calibrated (<20 ppm) using trypsin autolysis products. Post-acquisition baseline correction and smoothing was carried out using software provided with the instrument. Spectra were submitted to Mascot (<http://matrixscience.com>) for peptide mass fingerprinting. Surface enhanced laser desorption/ionization (SELDI)-TOF analysis was also performed. Purified LC was spotted on a normal phase ProteinChip array and allowed to air dry. The ProteinChip spot was washed with an excess of water and sinapinic acid (Agilent Technologies, CA) was applied and allowed to dry. Mass spectra were acquired with a PBS-IIc SELDI-TOF mass spectrometer (Ciphergen Biosystems, Fremont, CA) using the ProteinChip software provided with the instrument with the following instrument settings: laser power 220, sensitivity 8, focus mass 50,000.

Isoelectric focusing

The isoelectric point of BoNT/B LC was determined using the imaged capillary isoelectric focusing (cIEF) method with the iCE280 system from Convergent Bioscience [25]. Focusing was performed using 100 mM H_2PO_4 as anolyte, 100 mM NaOH as catholyte, a focusing voltage of 3 kV and a focusing time of 5 min. Initial standardization of the instrument was performed using hemoglobin standard from Convergent Bioscience (Toronto, Canada). A working ampholyte solution was prepared by mixing 80 μl (100%) of broad range ampholyte pH 3–10 (Amersham Biosciences, Piscataway, NJ) to 920 μl of 0.5% methyl cellulose solution. The sample mix was prepared by mixing 176 μl of this working ampholyte solution, 2 μl of prediluted pI marker 7.0 and 2.0 μl of marker 8.6 (Bio-Rad, Hercules, CA). Twenty microliters of the protein sample was added to this mixture to make a total volume of 200 μl . Injection volume was 40 μl and the exposure time and current settings were 45 ms and 8.75 A, respectively.

Focusing time was done for 7 min. Four runs were made and the initial values for the *pI*, obtained in pixels, were calibrated into *pI* using known markers, i.e., 7.0 and 8.6.

UV-visible absorption, circular dichroism, and fluorescence measurements

Before each experiment, to determine protein concentration and assess its purity UV-visible absorption spectra were recorded at 20°C with a Hewlett-Packard 8452 diode array spectrophotometer. LC concentration was determined using $A_{278}^{1\text{cm}}$ (1 cm light path) value of 1.0 at 278 nm [26]. Circular dichroism spectra were recorded at 20°C and temperature-dependent unfolding of LC was followed by monitoring circular dichroism (CD) at 222 nm on a Jasco J-810 spectropolarimeter with quartz cuvettes of 1 mm path length. To increase signal-to-noise ratio an average of five scans were recorded at a scan speed 20 nm/min with a response time of 4 s. For all measurements a buffer blank was recorded and subtracted from sample recordings. A MW of 50975 for the 441-residue LC yielded the mean residue weight as 115.59. Tryptophan fluorescence emission spectra were recorded at 20°C in a PTI QuantaMaster spectrofluorimeter, model RTC 2000 equipped with a Peltier controlled thermostat and Felix software package. Emission and excitation slit widths were set at 1 nm and excitation wavelength at 295 nm. An average of five scans was recorded for each spectrum. The solution properties of the peptide were investigated by CD [27–29].

Enzymatic activity assay of BoNT/B LC

Before each experiment, aliquots of the protein were passed through a PD-10 gel-filtration column equilibrated with 10 mM Na-phosphate, pH 6.5, to remove EDTA present in the storage buffer. The enzymatic activity assay was based on HPLC separation and measurement of the cleaved products from a 35-mer synthetic peptide corresponding to human VAMP-2 residues 60–94 (HV35) [27]. BoNT/B LC catalyzes the hydrolysis of this peptide between residues 17 (glutamine) and 18 (phenylalanine) corresponding to residues Q76 and F77 of human VAMP-2. Assay mixtures (30 μ l) containing 0.18 mM substrate, 50 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) pH 7.2, 0.25 mM ZnCl_2 , 5 mM DTT, and 1.0 μ g light chain were incubated at 37°C for 5 min. Assays were stopped by adding 90 μ l of 0.7% trifluoroacetic acid. The amounts of uncleaved substrate and the products were measured after separation by reverse-phase HPLC. Solvent A consisted of 0.1% trifluoroacetic acid and solvent B consisted of 70% acetonitrile/0.1% trifluoroacetic acid. The flow rate was 1.0 ml/min at 25°C and the gradient profile was as follows: 20% B (2.5 min); linear gradient to 80% B (21 min); 100% B (6 min). Kinetic parameters of the synthetic substrate were calculated from Lineweaver–Burke plot of initial rates of proteolysis

by BoNT/B LC versus various peptide concentrations ranging from 0.016 to 0.1 mM.

Fragmentation of recombinant light chain

BoNT/B LC was passed through a PD-10 column to remove EDTA and collected in 50 mM Na-phosphate pH 6.5. The LC was mixed in the presence or absence of 0.5 mM ZnCl_2 and aliquots (30 μ l) of the LC were distributed in screw-capped eppendorf tubes. The final concentration of the protein in each incubation tube was 0.35 mg/ml. The tubes were incubated at 4 and 23°C. At specified time intervals 20 μ l of 2× SDS-load buffer was added to a 30 μ l aliquot for SDS-PAGE analysis.

Immunogenicity studies

Three groups of 20 mice were used for each protection assay. Survivor data is recorded as the number of survivors from the total number of animals tested. Animals used for the study were female Crl:CD-1 mice, 16–22 g, on receipt (Charles River, Wilmington, MA). Groups were inoculated three times at 0, 2, and 4 weeks, with 5 μ g or 15 μ g of immunogen (BoNT/B LC) per mouse. Injection volume was 100 μ l per mouse. Intramuscular injection into the caudal thigh muscle mass with 0.2% Alhydrogel in 0.8% saline with 0.8% benzyl alcohol as a preservative was performed. Intra-ocular bleeds were performed on anesthetized mice to collect sera 2 days after the last injection. Mice were challenged 7 days after the final inoculation with 10^2 MLD₅₀ or 10^3 MLD₅₀ BoNT-B toxin from *C. botulinum* strain Okra (Metabio, Madison, WI) in gel-phosphate buffer (0.4% dibasic phosphate with 0.2% gelatin). Naïve mice were challenged with the same levels of toxin and used as control. Mice were observed daily and the number of mice that survived after 5 days was recorded. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principals stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where the research was conducted is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International.

Enzyme-linked immunosorbent assay (ELISA)

Immulon 2 plates (Dynatech, Chantilly, VA) were coated with botulinum neurotoxin type B (Metabio, Madison, WI) at 2 μ g/ml, 100 μ l/well, in phosphate-buffered saline (PBS) pH 7.4. The plates were incubated overnight in a humidity box at 4°C. Diluted serum was added in duplicate to toxin-coated wells (100 μ l/well). The secondary antibody was horseradish peroxidase conjugated goat anti-mouse IgG mAb and ABTS substrate was added as color developer (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The absorbance was measured with a microplate reader at 405 nm. Naïve mouse serum was added as a

negative control in each assay. The titer was defined as the reciprocal of the last dilution with an absorbance ≥ 0.2 above background absorbance.

Results and discussion

Construction of the BoNT/B LC expression system

Previously, tetanus toxin fragment C had been expressed in *E. coli* at 3–4% cell protein. The sequence for *Clostridium tetani* was examined and it was found to contain rare *E. coli* codons encoding fragment C. When the coding sequence was replaced by sequence optimized for codon usage in *E. coli*, it had been shown that the expression of fragment C is increased approximately 11–14% [31]. Others have used a strong promoter of phage T₇ and physiological control to improve the level of expression of a synthetic BoNT/B LC [32]. In the present study we utilized both codon optimization, which was performed to limit reduced protein expression associated with rare codons and high AT base composition [33,34], and a strong T₇ promoter. The use of a strong promoter and optimized synthetic BoNT/B LC gene resulted in a 50% higher yield of protein expression and/or recovery than previously reported [32]. We produced a recombinant protein in a correctly folded state that was biologically active. Primers for amplifying the nucleotide sequence encoding the BoNT/B LC were constructed on the basis of the Minton sequence [35]. Oligonucleotides were designed to contain *Nde*I–*Bam*HI restriction sites that facilitated the insertion of the 1323 nucleotide sequence in frame with the pET24a+ parent vector beginning with an ATG start codon. At the protein level the sequence shared 100% identity to the Minton sequence.

Expression and purification of the BoNT/B LC

Upon induction by the addition of IPTG at 18°C, BoNT/B LC was over-expressed. The recombinant protein was solubilized in lysis buffer before sonication to reduce the amount of cell debris and nucleic acid. A clear lysate was obtained after centrifugation which represents the crude protein solution and contains the BoNT/B LC in the soluble fraction. The soluble protein was purified to near homogeneity by two rounds of cation exchange chromatography using a Poros HS 20 followed by a third pass through cation exchange chromatography using a Source S column (Figs. 1A and B); peak fractions were collected and analyzed for purity. Verification of the purified 50 kDa protein was done by SDS–PAGE using Coomassie brilliant blue R-250 stain (Fig. 2A) and SilverXpress silver stain (Fig. 2B) and Western blot analysis using a horse polyclonal antibody (Fig. 2C). The efficiency of the purification process is shown in Table 1. Protein obtained from a 1 L culture typically yielded ≥ 12 mg which was greater than 98% pure based on densitometry analysis of the silver stained gel.

N-terminal sequence and mass spectroscopic analysis of rBoNT/B LC

The molecular mass of the purified recombinant BoNT/B LC was determined by surface enhanced laser desorption/ionization (SELDI)-TOF analysis. It was 50.8 kDa which corresponded to the predicted molecular mass based on the amino acid sequence. Matrix assisted laser desorption/ionization (MALDI)-TOF was also performed and 85% of the amino acid sequence was observed. To verify whether the LC contains the initiating methionine residue, N-terminal sequencing analysis was performed on the first 10 residues of the protein. The N-terminal amino sequence of recombinant BoNT/B LC was PVTINNFNYN as expected. We observed that the initiating methionine residue of the protein had been removed by *E. coli* methionyl aminopeptidase.

Isoelectric focusing

The isoelectric focusing analysis of the recombinant BoNT/B LC revealed a pI of 7.25 which is higher than the calculated theoretical isoelectric point of 6.3 (<http://us.expasy.org>). The experimental pI value for the native light chain was equal to the calculated pI 6.3. A number of methods have been proposed for the theoretical determination of the pIs of proteins [36,37]. Typically, these methods give results that are within ± 1 pH unit of the experimental pI. When calculating theoretical pI values, calculated pIs often disagree with experimentally measured pIs [38]. The theoretical pI estimate of 6.3 assumes that all residues have pK_a values that are equivalent to the isolated residues. The actual pI value for a protein is affected by the tertiary structure of the protein, which can lead to differences between calculated and experimental pI values. A change in the pI value (6.3–7.25) may be the result of masking of carboxyl groups in the tertiary structure of the protein [39].

Structural features of LC at different pH values and in the presence of zinc

To detect and examine any conformational changes induced by pH differences, buffer and zinc, we employed far-UV circular dichroism and tryptophan fluorescence emission spectroscopies in the absence and presence of 0.5 mM ZnCl₂ in 46 mM Pipes, pH 6.5, and in the absence of ZnCl₂ in 46 mM Mes, pH 5.5 or 46 mM Hepes pH 8.0 (Fig. 3). The CD spectra were similar in the four conditions displaying the characteristic double minima at 208 and 220 nm as expected (Fig. 3A). This indicates that the secondary structure of BoNT/B LC was similar in all conditions. The tryptophan fluorescence emission spectra were similar in all conditions tested. However, the addition of 0.5 mM ZnCl₂ increased the fluorescence intensity about 11% as compared to the fluorescence intensity at pH 6.5 without affecting the emission maximum or shape of the spectrum (Fig. 3B). The fluorescence intensity decreased

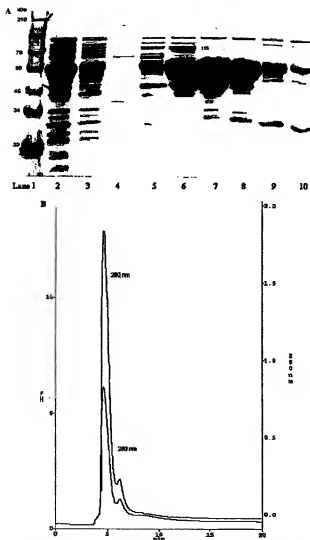


Fig. 1. (A) First round purification of BoNT/B LC from *E. coli*. Recombinant BoNT/B LC was purified from *E. coli* cell paste using a three column strategy described in Results. Protein samples were separated by SDS-PAGE and visualized by Coomassie stain. Invitrogen's pre-stained SeeBlue molecular weight markers were used (15 μ l lane 1). Clarified crude lysate (lane 2) was loaded onto Poros HS 20 column. Lane 3 represents the flow through, lanes 4 and 5 represent fraction 21 and 22, respectively. Lanes 6, 7, and 8 represent fractions 23, 24, and 25 respectively which were pooled and subjected to another round of cation exchange chromatography. Lane 9 represents fraction 26 and lane 10 represents fraction 27. (B) Purification chromatogram for the recombinant protein LC column Poros HS 20.

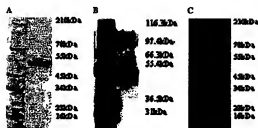


Fig. 2. (A) Reducing SDS-PAGE of purified BoNT/B LC separated on a 10% tricine gel and visualized by Coomassie blue stain; Invitrogen's pre-stained SeeBlue molecular weight markers were used (15 μ lane 1). (B) Silver stain analysis of purified BoNT/B LC following SDS-PAGE. (C) Western blot analysis of purified BoNT/B LC detected with polyclonal horse BoNT/B antibody.

Table 1

Purification of recombinant BoNT/B LC from the soluble fraction

| Steps | Total protein (mg) ^a | Step yield (%) | Overall yield (%) |
|-------------------------------|---------------------------------|----------------|-------------------|
| Soluble fraction | 91 | 100 | 100 |
| 1st ion exchange HS 20 | 16 | 17 | 17 |
| 2nd ion exchange HS 20 | 10 | 80 | 9 |
| 3rd ion exchange sources 15 S | 4 | 95 | 4 |

Data are normalized to quantities obtained per gram of cell paste.

^a Total protein was determined by BCA assay (Pierce) with BSA as a standard.

about 11% as compared to the fluorescence intensity at pH 6.5 without affecting the emission maximum or shape of the spectrum when the protein was at high pH in the absence of $ZnCl_2$ (Fig. 3B). The blue-shifted tryptophan fluorescence emission spectra with a λ_{max} of 318 nm (λ_{max} of free tryptophan is about 354 nm) suggests that the single tryptophan residue (W44) is buried in a hydrophobic environment that is not significantly affected by either pH or zinc. The results of the CD and fluorescence experiments suggest that pH changes over the range of 6.5–8.0 and the addition of 0.5 mM $ZnCl_2$ have no significant effect on secondary and tertiary structures of BoNT/B LC.

We can also conclude from the data that the LC remains stable up to 40°C with pH values in the range of 6.5–8.0. Estimation of the secondary structure at 20°C represents the native structure of the LC. Alterations in the secondary structure content of BoNT/B LC at 20°C with respect to various conditions are listed in Table 2. When the pH rises there is a marginal increase in the α -helix content of the LC and a marginal decrease in the β -sheet content is observed. The addition of zinc also appears to increase the α -helix content of the LC as well as decrease the β -sheet content. There is no significant change in the content of turns or random coils of the LC under all conditions tested.

Properties of the zinc-metalloprotease activity of the BoNT/B LC

In order to determine the optimum conditions for the metalloprotease activity of BoNT/B LC, several parameters

were examined (Fig. 4). The pH dependence on the cleavage of 35-residue peptide of human VAMP-2 (HV35) by BoNT/B LC was assessed using three buffers, Mes, Pipes, and HEPES (Fig. 4A). Optimal activity of the protease activity of BoNT/B LC was found at near-neutral pH with a maximum between 6.6 and 7.3 (Fig. 4A). The rate of cleavage of peptide HV35 was markedly reduced at pH values higher than 7.5 and lower than 6.0. Although pH variations could cause changes in the substrate diminishing the cleavability in a pH-dependent fashion, the small level of activity remaining at pH values higher than 7.5 and lower than 6.0 could be accounted for by a change in the ionization of the histidine residues which co-ordinate the zinc molecule in the catalytic mechanism of BoNT/B LC. Fig. 4A also demonstrates that the rate of cleavage of HV35 by BoNT/B LC was influenced by the type of buffers used in the reaction mixtures. Cleavage rates of the peptide in PIPES were lower than that observed with HEPES and MES. For comparison, the optimum pH values of BoNT/A LC [40] and BoNT/B [41] are 7.0–7.5 and 6.5–7.0, respectively. Since the highest rates of metalloprotease activity by BoNT/B LC were observed in HEPES buffer, this buffer was used throughout assay experiments. The metalloprotease activity of BoNT/B LC was also influenced by temperature; being more active at 37°C (Fig. 4B). Similar results were noted for BoNT/A LC and BoNT/B. It is uncertain whether this result is a reflection of the thermal conformational stability of BoNT/B LC or substrate HV35. The effect of ionic strength on metalloprotease activity of BoNT/B LC was also studied. Increasing the concentration of sodium chloride in 50 mM HEPES buffer had markedly inhibited the protease activity of BoNT/B LC (Fig. 4C). Similar results with respect to the effect of ionic strength have been reported for the cleavage of HV35 by BoNT/B [30].

Thermal denaturation of BoNT/B LC

To probe the pH and $ZnCl_2$ affect on LC stability, unfolding of LC was examined by monitoring the CD signal at 222 nm as a function of temperature. Fig. 5 shows the thermal transition curves of LC under different

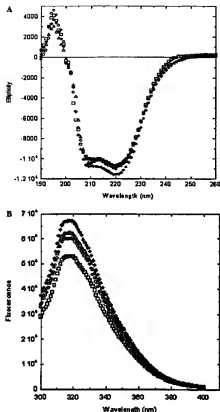


Fig. 3. Far UV circular dichroism (A) and tryptophan fluorescence emission (B) spectra of BoNT/B LC in the absence (circle) and presence of 0.5 mM ZnCl_2 (closed diamond) in 46 mM Pipes, pH 6.5, and in the absence of ZnCl_2 in 46 mM Mes, pH 5.5 (triangle) and in 46 mM Hepes, pH 8.0 (open square). Protein concentration in these experiments was 0.15–0.19 mg/ml.

Table 2

Calculated secondary structural content of BoNT/B LC under various conditions

| Buffer | pH | Addition | % α -helix | % β -sheet | % turns | % random coil |
|--------|-----|-----------------|-------------------|------------------|---------|---------------|
| Mes | 5.5 | None | 16.5 | 31 | 28.5 | 24 |
| Pipes | 6.5 | ZnCl_2 | 20 | 26.5 | 29 | 24.5 |
| Pipes | 6.5 | None | 18.5 | 28 | 29.5 | 24 |
| Hepes | 8.0 | None | 18 | 29 | 29 | 24 |

Secondary structural contents were calculated by SELCON using the Sotsec program (Softwood).

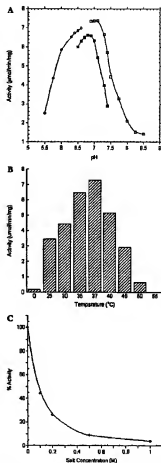


Fig. 4. Effect of pH, temperature, and ionic strength on the metalloprotease activity of BoNT/B LC. (A) The pH dependence on the cleavage of the synthetic, VAMP-derived substrate (HV35) by BoNT/B LC was assessed using three buffers, Mes (circle), Pipes (closed square), and Hepes (open square). Assays contained 0.2 mM peptide substrate, 1.0 μg LC and 50 mM buffer at various pH values. Each data point represents an average of three determinations. (B) Reaction mixtures (0.03 mL) containing 50 mM Hepes pH 7.2, 0.18 mM substrate, and 1.0 μg BoNT/B LC incubated at the indicated temperature for 5 min. Each point was performed in triplicate and the maximum activity (100%) was 7.4 $\mu\text{mol/min/mg}$ LC at 37°C. (C) Reaction mixtures (0.03 mL) containing 50 mM Hepes, pH 7.2, 0.18 mM substrate, 1.0 μg BoNT/B LC, and the appropriate NaCl concentrations were incubated at 37°C for 5 min. Each point was performed in triplicate and the maximum activity (100%) was 7.3 $\mu\text{mol/min/mg}$ LC in the absence of NaCl.

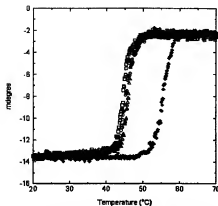


Fig. 5. Thermal unfolding of BoNT/B LC in the absence (circle) and presence of 0.5 mM ZnCl_2 (closed diamond) in 46 mM Pipes pH 6.5 and in the absence of ZnCl_2 in 46 mM Mes pH 5.5 (triangle) and in 46 mM Hepes (open square) at 222 nm. Protein concentration in these experiments was 0.15–0.19 mg/ml. T_m (the midpoint of thermal transition) values were calculated as 46 °C at pH 5.5, 6.5, and 8.0 in the absence and 56 °C at pH 6.5 in the presence of 0.5 mM ZnCl_2 .

pH conditions and in the presence of 0.5 mM ZnCl_2 . Although the secondary structure was not affected by zinc and different pH conditions, the unfolding pattern of LC determined in the presence of ZnCl_2 differed significantly. Sharp and monophasic denaturation curves were obtained in the absence and presence of 0.5 mM ZnCl_2 in 46 mM Pipes, pH 6.5, and in the absence of ZnCl_2 in 46 mM Mes, pH 5.5, or 46 mM Hepes, pH 8.0, indicating that the LC preparation was homogeneous in all conditions. The thermal denaturation curves allowed calculation of the apparent melting temperature T_m (midpoint of thermal transition) values. The denaturation curves of the LC in the absence of ZnCl_2 at pH 5.5, 6.5, and 8.0 coincided and yielded a T_m of 46 °C. LC at pH 5.5, 6.5, and 8.0 in the presence of ZnCl_2 yielded a T_m of 56 °C. We can conclude from this data that the presence of ZnCl_2 appears to significantly stabilize the LC as indicated by an increase in T_m by 10 °C.

Enzyme kinetics of BoNT/B LC

To determine the K_m and V_{max} values initial rates of proteolysis were determined in triplicate for various concentrations of the substrate (0.016–0.1 mM) and results were plotted as $1/V$ versus $1/S$ (Fig. 6, Lineweaver–Burke plot). Under the conditions of the assays, the recombinant light chain cleaved the substrate with a K_m of 0.083 mM, a V_{max} of 7.8 $\mu\text{mol}/\text{min}/\text{mg}$ LC, and the K_{cat} was determined to be 40/s. For comparison, the K_m of the native dichain toxin is reported to be 0.33 mM ($k_{cat} = 24/\text{s}$) [30]. The lower K_m for

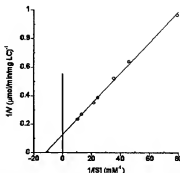


Fig. 6. Determination of K_m and V_{max} from the double-reciprocal (Lineweaver–Burke) plot of initial rates of proteolysis versus seven different substrate concentrations ranging from 0.016 to 0.1 mM by BoNT/B LC. Assay mixtures (30 μl) contained 50 mM HEPES buffer (pH 7.2) and 1.0 μg of BoNT/B LC. The K_m and V_{max} were calculated as 0.083 mM and 7.8 $\mu\text{mol}/\text{min}/\text{mg}$ LC, respectively.

the LC may be due to a more exposed active site in the free LC than in the LC of the native toxin where the active site is surrounded by the translocation domain belt and the long axis of the translocation domain [42]. Thus, the catalytic efficiency k_{cat}/K_m of the LC, 482 $[(\text{M}^{-1}\text{s}^{-1}) \times 10^3]$ which is consistent with the reported value [43], is higher than that of the native toxin, 72 $[(\text{M}^{-1}\text{s}^{-1}) \times 10^3]$ [30].

Zinc-enhanced fragmentation experiments

To test the stability of the protein, as well as examine the protein for fragmentation similar to that of BoNT/A LC as reported previously [40], protein was stored in aliquots and left at 23 or 4 °C for a predetermined time. SDS–PAGE load buffer was added to an aliquot before running the protein on a 10% tricine gel. The protein stored at 4 °C was stable for a period of 110 days in the presence or absence of ZnCl_2 and no fragmentation was observed (not shown). However, the protein stored at 23 °C demonstrated truncation of the LC when the protein was incubated in the presence of ZnCl_2 (Fig. 7) at day 25. Examination of BoNT/B LC stored with ZnCl_2 at room temperature for a shorter period of time indicated that fragmentation can be detected. Similar results have been reported for BoNT/A LC [26].

We also tested the activity of the protein at the end point and found that the protein stored in 50 mM sodium phosphate, pH 6.5 at 4 °C retained 100% of its initial catalytic activity while the protein stored at 23 °C, in the same buffer, retained 90% of its enzymatic activity.

Immunogenicity studies

Animal protection studies were done to determine if the purified recombinant BoNT/B LC has the ability to elicit



Fig. 7. BoNT/B LC in 50 mM Na-phosphate buffer pH 6.5 in the presence and absence of 0.5 mM $ZnCl_2$ was aliquoted and left at 23 °C temperature for 25 days. Lane 1, Invitrogen's Mark12 molecular weight marker (15 μ l); lane 2, day zero, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the absence of $ZnCl_2$; lane 3, day zero, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the presence of $ZnCl_2$; lane 4, day 25, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the absence of $ZnCl_2$; lane 5, day 25, BoNT/B LC in 50 mM Na-phosphate buffer, pH 6.5, in the presence of $ZnCl_2$; lane 6, Invitrogen's SeeBlue molecular weight marker (15 μ l); SDS-PAGE load buffer was added to the sample before running on a 10% tricine gel. Protein stored at 23 °C demonstrated truncation of the LC when the protein was incubated in the presence of $ZnCl_2$ (lane 5) at day 25.

protective immunity in mice. Three groups of 20 mice were used for each protection assay. Two groups of 20 mice were injected with the LC i.m.; one group with 5 μ g/mouse and one group with 15 μ g/mouse. Each group received three injections, were bled, and subsequently challenged with BoNT B toxin by i.p. administration (Table 3). Within each group of 20, 10 mice were challenged with 1×10^2 and 10 mice were challenged with 1×10^3 MLD₅₀ holotoxin. Survivor data is recorded as the number of survivors from the total number of animals tested. Naïve mice were challenged with the same levels of toxin and used as control. Mice were observed daily and the number of mice that survived after 5 days was recorded. None of the control animals survived when toxin was administered. Mice that received three injections of 5 μ g of purified soluble BoNT/B LC were completely protected when challenged with i.p. 10^2 or 10^3 LD₅₀ of BoNT/B toxin. Three doses of 15 μ g of purified BoNT/B LC protected mice from a challenge of 10^2 or 10^3 LD₅₀ of

Table 4

Correlation of individual ELISA titers with survival after vaccination with purified recombinant BoNT/B LC

| Individual ELISA titer ^a | Survival (no. alive/total) ^b | % survival |
|-------------------------------------|---|------------|
| ≤ 100 | 27/28 | 96 |
| 400 | 25/25 | 100 |
| ≥ 1600 | 7/7 | 100 |

^a Serum was bled from each mouse individually. Titer is the reciprocal of the highest dilution having an A_{490} of greater than 0.2 after correction for background.

^b Mice were challenged with 10^2 or 10^3 i.p. LD₅₀ of BoNT/B 7 days after the last vaccination.

native BoNT/B toxin with the exception of one mouse (Table 4). Individual serum antibody ELISA titers of mice injected with soluble BoNT/B correlated with survival as all mice with ELISA titers of 100 or greater survived toxin challenge (Table 4). Mice with titers less than 100 did not survive. The work presented here demonstrates that purified BoNT/B LC has the capacity to protect against a challenge dose of neurotoxin.

Conclusions

This paper describes the expression of BoNT/B LC in *E. coli* using an optimized synthetic gene to produce a highly pure, 50 kDa protein. The growth and induction conditions for expression were optimized to obtain this protein in the soluble fraction. Traditional ion exchange chromatography methods were used to purify a protein that is highly active. CD analysis and tryptophan fluorescence emission spectroscopy indicate that the LC possesses a defined set of secondary and tertiary structures. The only Trp residue is in a hydrophobic environment as indicated by a blue-shifted emission λ_{max} of 318 nm. The CD analysis indicates that the secondary structure of the light chain consists predominantly of β -sheets which is consistent with the known structure of the recombinant BoNT B heavy chain [44]. We also conclude that the Trp residue is constrained in the protein hydrophobic core, a feature supported by the published X-ray crystallography structure of BoNT/B [42,45]. The unfolding of BoNT/B LC at different pH values demonstrate that pH has no significant effect, however, the addition of zinc increases the thermal stability of the LC at pH 6.5. We have also demonstrated that 50 mM Hepes, pH of 7.2–7.3 is the best system among the buffer systems tested to obtain a maximum enzymatic activity. Kinetic analysis demonstrated that the catalytic efficiency of the LC is higher than that of the native dichain toxin. This may indicate that the LC has a more exposed active site in free LC than in the LC of the native toxin where the active site is surrounded by the translocation domain belt and the long axis of the translocation domain [42]. The purified BoNT/B LC was stable for at least 110 days when stored at 4 °C in 50 mM sodium phosphate, pH 6.5, remained fully soluble, and retained its initial catalytic activity.

Table 3
Survival of challenged mice vaccinated with BoNT/B LC from *E. coli*

| Mice | Challenged with 10^2 MLD ₅₀ BoNT/B survivors/challenged | Challenged with 10^3 MLD ₅₀ BoNT/B survivors/challenged |
|------------------------------|--|--|
| Group I _(total) | 10/10 | 10/10 |
| Group II _(total) | 9/10 | 10/10 |
| Group III _(naïve) | 0/10 | 0/10 |

Mice in group I received 5 μ g of purified recombinant BoNT/B LC adsorbed to Alhydrogel, injections were at 0, 2, and 6 weeks. Group II received 15 μ g of purified recombinant BoNT/B LC adsorbed to Alhydrogel, injections were at 0, 2, and 6 weeks. Group III were naïve mice. Mice were immunized and challenged by administration of neurotoxin. Mice were observed daily and the number of mice that survived after 5 days was recorded.

Like BoNT/A LC, BoNT/B LC expressed and purified from *E. coli* is nontoxic when injected into mice at a concentration of 5–15 µg of LC per mouse [40]. Table 4 shows that all mouse antisera tested had titers against BoNT/B. With the exception of one mouse, which had a titer below 100, the mice were protected against subsequent challenge with low doses of BoNT/B toxin. In contrast, purified BoNT/A LC failed to protect even when titers were boosted with adjuvant [40].

In conclusion we have expressed and purified ≥4 mg/g quantities of recombinant BoNT/B LC and characterized the protein. The protein is soluble, nontoxic, catalytically active and highly stable making it ideal for investigators to identify potential inhibitors.

Acknowledgments

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Roads From Vaccines to Therapies

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Abstract: Over the past decade, we have demonstrated that various recombinant fragments of botulinum neurotoxin are highly immunogenic, stimulating notable levels of protective antibodies in mice, guinea pigs, and nonhuman primates. One of the fragments evaluated, the fragment C, is a potential next-generation vaccine candidate to replace the current pentavalent botulinum toxoid vaccine. Synthetic genes encoding the carboxyl-terminal regions (~30 kDa) of toxin types A, B, C1, E, and F were expressed in *Pichia pastoris*, and manufacturing processes were developed for producing highly purified vaccines. These vaccines were shown to be safe, highly efficacious, stable, and amenable to high-level industrial production. Recombinant vaccines are now being produced in accor-

dance with current Good Manufacturing Practices for use in future clinical trials. As our discovery-based program on vaccine development is diminishing, it is concurrently being replaced with a program focused on developing therapeutic interventions to botulism. Synthetic genes encoding the light chains of botulinum toxin have been expressed in *Escherichia coli*, and purified. These proteolytically active light chains are being used in high-throughput assays to screen for inhibitors of its catalytic activity. Other resources developed as part of the vaccine initiative, likewise, are finding utility in the quest to develop therapies for botulism. © 2004 Movement Disorder Society

Key words: botulinum; neurotoxins; vaccines; therapies

Botulism, a potentially fatal paralytic disease, is caused by highly potent neurotoxins^{1,2} produced by the Gram-positive, anaerobic spore-forming bacterium *Clostridium botulinum*. *C. botulinum* produces seven forms of antigenically distinct exotoxins, which are differentiated serologically by specific neutralization.³ They have been designated as serotypes A, B, C1, D, E, F, and G. Polyclonal antibodies derived for a specific neurotoxin can neutralize the toxic effects of that toxin but will not cross-neutralize another toxin serotype.

Presently, there are no licensed vaccines for preventing botulism. A limited supply of pentavalent botulinum toxoid vaccine, manufactured in the 1970s by the Michigan Department of Public Health, is avail-

able under Investigational New Drug status for administration to individuals considered to be "at risk" for toxin exposure. Except for the human Botulinum Immune Globulin product used in infant botulism cases against type A and B toxins, therapeutic intervention of botulism is limited to equine-based antitoxin treatment and concurrent intubation and ventilatory assistance for respiratory failure.

Over the past 10 years, we have been involved in developing recombinant vaccines against the botulinum neurotoxins (BoNT). Early in discovery, the non-toxic fragment C (H_c) region of the toxin was shown to be a suitable vaccine candidate because of its ability to elicit high levels of protective immunity in animal models. Genetic engineering technologies were used to optimize gene expression and increase protein stability. Recombinant vaccines have now been produced for BoNT serotypes A, B, C1, E, and F.⁴⁻⁹ These vaccine candidates are stable, safe, and highly effective at eliciting short and long-term immunity in the animals tested.

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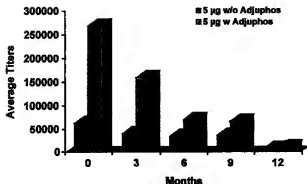


FIG. 1. Average anti-botulinum neurotoxin serotype A antibody titers over 12 months.

Recently, we expanded our discovery-based efforts to develop therapeutic interventions to botulism applying many of the same concepts and resources used in the vaccine effort. This study describes the antigenic properties of BoNT fragments, and the production of recombinant light chains for use in high-throughput assays to screen for antagonists to its protease activity.

MATERIALS AND METHODS

Production of Recombinant BoNT Domains

Synthetic gene sequences for BoNT light chain (LC), translocation domain (H_L), and fragment C (H_C) were constructed to lower the A+T base content and remove rare codons specific to *C. botulinum*. Genes were subsequently cloned and expressed in *Escherichia coli* or *Pichia pastoris*, and their gene products were purified by using conventional ion exchange chromatography alone or in concert with hydrophobic chromatography.

Efficacy Assays

Efficacy assays were performed as previously described.⁹ Briefly, groups of mice (Crl-CD-1; ICR) were inoculated IM with one to three doses of purified recombinant BoNT antigen at intervals of 0, 2, and 4 or 0, 4, and 8 weeks. Vaccine was adsorbed to 0.2% Alhydrogel unless otherwise stated. Mice were challenged IP with 10^5 to 10^6 mouse IP LD₅₀ of toxin. A control group of unvaccinated mice challenged with 10^5 LD₅₀ of toxin was used for each separate experiment. Challenges were 14 days after the final inoculation. Animal studies were conducted in compliance with the Animal Welfare Act and other Federal statutes and regulations relating to

animals and experiments involving animals and adhere to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

Total and Protective Serum Antibodies

Mice were bled retroorbitally while anesthetized 2 days before challenge. Serum was coded against the mice to compare survival versus enzyme-linked immunosorbent assay (ELISA) titer (i.e., total antibody titer). Twenty-five microliters of serum from mice of each set of vaccinations was pooled and used to determine the average serum neutralization titer after each inoculation (i.e., protective antibody titer). ELISA and mouse neutralization assays were previously described.⁹

Assay for Protease Activity

Enzymatic assays were performed as previously described.^{10,11}

RESULTS

Recombinant BoNT/A (H_L), initially expressed and purified from *E. coli*, was used in animal protection studies to determine its ability to elicit both short and long-term immunity in mice. Groups of 10 mice received three doses (5 µg per dose) of vaccine, in the presence or absence of an aluminum phosphate adjuvant. Total antibody titers (Fig. 1) were determined for the groups of mice at various time intervals after vaccination. Mice inoculated with vaccine adsorbed to Adjuvophos adjuvant

TABLE 1. Survival of challenged mice vaccinated with BoNT/A (H_2) from *Escherichia coli*

| Mice | Challenged with 10^5 MLD ₅₀ BoNT/A at various times after initial vaccination (mo.) | | | | |
|----------|--|-------|-------|-------|-------|
| | 1.5 | 3 | 6 | 9 | 12 |
| Group I | 10/10 | 10/10 | 9/9 | 10/10 | 10/10 |
| Group II | 10/10 | 10/10 | 10/10 | 9/10 | 8/8 |
| Controls | 0/10 | 0/10 | 0/10 | 0/10 | 0/10 |

Mice in group I received 5 μ g of purified recombinant BoNT/A (H_2) adsorbed to aluminum phosphate at 0, 2, and 4 weeks. Mice were immunized by intramuscular injection and challenged by intraperitoneal administration of neurotoxin. Group II received 5 μ g of purified recombinant BoNT/A (H_2) in saline at 0, 2, and 4 weeks. Mice were immunized by intramuscular injection and challenged by intraperitoneal administration of neurotoxin. Controls were naive mice. BoNT, botulinum neurotoxin; (H_2), non-toxic fragment C.

(0.175%) had higher total antibody levels than mice receiving vaccine without adjuvant. Antibody titers were detectable by ELISA up to 12 months after vaccination (Fig. 1). These titers, although significantly lower at 12 months after vaccination, completely protected mice from 10^5 MLD₅₀ of a BoNT/A challenge (Table 1).

Fragment C vaccines for serotypes A, B, C, E, and F toxins were subsequently expressed and purified from the methylotrophic yeast *Pichia pastoris*. All five vaccines were tested for their ability to induce protective immunity in mice. Mice were given one, two, or three doses (1 μ g per dose) of vaccine at 0-, 4-, and 8-week intervals, then challenged with increasing levels of homologous toxin (Table 2). All mice receiving two doses of vaccine

were completely protected from a challenge of 10^5 MLD₅₀ toxin regardless of the BoNT serotype (Table 2). All mice receiving three vaccinations, except for mice receiving rBoNT/F (H_2) vaccine and challenged with BoNT/F, were completely protected from a toxin challenge of 10^5 MLD₅₀. Mice covaccinated with rBoNT/B (H_2) and rBoNT/F (H_2) vaccines were protected from a challenge of 10^5 MLD₅₀ BoNT/F (Table 2).

BoNT/A light chain (LC), LC+belk, and LC+translocation domain (H_2) were expressed and purified from the soluble fraction of an *E. coli* cell lysate. Recombinant fragments were assayed for enzymatic and immunological activities. The specific activity of purified rLC in a protease assay using a SNAP-25 derivative (synaptosomal-associated protein of Mr = 25 kDa) substrate was 2.15 μ mol/min per mg; LC+belk, 0.27 μ mol/min per mg; and LC+ H_2 , 0.02 μ mol/min per mg (Table 3). LC and LC variants were evaluated for their ability to elicit protective immunity in mice. Recombinant LC, purified and refolded from inclusion bodies, as well as LC in acetate buffer failed to induce protective antibodies against active toxin in mice (Table 4). LC in phosphate buffer, LC+belk, and LC+ H_2 were all able to induce protection, with LC+ H_2 having a higher potency than LC alone.

DISCUSSION

Advances in immunology and protein engineering have allowed the discovery and production of recombinant subunit vaccines. The epitopes recognized by neu-

TABLE 2. Immunological response of *Pichia pastoris*-produced BoNT (H_2) in mice

| Serotype | Doses (n) | % Survival against direct challenge* (LD ₅₀) | | | | GM ELISA titer | Serum neutralization (IU/ml) |
|------------------|-----------|--|--------|--------|--------|----------------|------------------------------|
| | | 10^3 | 10^4 | 10^5 | 10^6 | | |
| BoNT/A (H_2) | 1 | ND | 100 | 90 | 60 | 615 | 1.16 |
| | 2 | ND | 100 | 100 | 100 | 29,548 | 20.97 |
| | 3 | ND | 100 | 100 | 100 | 97,776 | 79.20 |
| BoNT/B (H_2) | 1 | 100 | 90 | 100 | ND | 1,678 | 0.13 |
| | 2 | 100 | 100 | 100 | ND | 89,144 | 28.67 |
| | 3 | 100 | 100 | 100 | ND | 97,776 | 28.67 |
| BoNT/C (H_2) | 1 | 90 | 50 | 70 | ND | 4,032 | 0.11 |
| | 2 | 100 | 100 | 100 | ND | 373,442 | 284 |
| | 3 | 100 | 100 | 100 | ND | 470,507 | 451 |
| BoNT/E (H_2) | 1 | 20 | 30 | 0 | ND | 138 | 0.06 |
| | 2 | 89* | 100 | 80 | ND | 74,100 | 1.41 |
| | 3 | 100 | 100 | 100 | ND | 242,000 | 48.20 |
| BoNT/F (H_2) | 1 | 100 | 90 | 0 | ND | <100 | <0.05 |
| | 2 | 80 | 100 | 0 | ND | 10,640 | 23.74 |
| | 3 | 100 | 100 | 0* | ND | 24,405 | 70.66 |

*Ten mice per group.

*There were 8/9 survivors.

*Survival was 10/10 at 100,000 LD₅₀ after covaccination with BoNT B (H_2).

BoNT/A-C, F; botulinum serotypes A-C and F; (H_2), non-toxic fragment C; ELISA, enzyme-linked immunosorbent assay; ND, not determined.

TABLE 3. Specific activity ($\mu\text{mol}/\text{min}/\text{mg}$)^a of LC, LC+belt, and LC+H₂ with a synthetic peptide substrate

| Construct | Activity ($\mu\text{mol}/\text{min}/\text{mg}$) ^a | Activity (relative) ^{ab} |
|---|--|-----------------------------------|
| LC-Naacetate ^b | 2.15 \pm 0.0113 | 1.00 |
| LC-phosphate ^b | 1.94 \pm 0.1138 | 0.90 |
| LC+belt ^b | 0.27 \pm 0.0053 | 0.15 |
| LC+H ₂ refolded ^{c,d} | 0.09 \pm 0.0073 | 0.08 |
| LC+H ₂ run ^{1c} | 0.02 \pm 0.0018 | 0.02 |

^aResults on activity are the average of three assays.^{ab}Activities adjusted for the respective molecular weights (MW) of the LC+belt (MW, 63.41 kDa) and LC+H₂ (MW, 98.69) relative to the LC (MW, 51.45 kDa).^bAssayed in the presence of Zn or DTT.^cThe protein was incubated with 7 mM DTT at room temperature for 17 min with the reaction started by adding substrate.^dThe protein was incubated with 7 mM DTT at room temperature for 17 min with the reaction started by adding ZnCl₂ and substrate.^eThere were at least five peaks in the HPLC run in addition to the two known product peaks of BoNT/A (LC) action. The additional peaks were generated from the substrate peptide in a time-dependent fashion, suggesting they were products of typical action on the substrate due to the presence of contaminating trypsin.LC, light chain; H₂, translocation domain; Zn, zinc; DTT, dithiothreitol; HPLC, high performance liquid chromatography.

tralizing antibodies can be found on fragments of a toxin molecule or on one or more proteins present on the surface of pathogenic organisms. We have produced recombinant subunit vaccines against botulinum neurotoxin types A, B, C, E, and F using a recombinant carboxyl-terminal fragment from each of the toxins. Due to manufacturing difficulties in producing large quantities of soluble BoNT (H₂) in *E. coli*, alternate expression systems were evaluated. *P. pas-*

toris was successfully used, and high-level expression of soluble BoNT (H₂) was achieved.⁴⁻⁹ Animal efficacy studies demonstrated that the fragment C vaccines elicit remarkable levels of protective immunity and that protection is long lasting.

BoNT/A (LC), LC + belt, and LC + H₂ were expressed in *E. coli*. By varying fermentation conditions, LC could be expressed as inclusion bodies or as a soluble protein. LC purified from inclusion bodies and refolded had a specific activity of 0.29 $\mu\text{mol}/\text{min}$ per mg¹⁰ comparable to that reported for native toxin¹² using a SNAP-25 derived substrate. LC expressed and purified from the soluble fraction of *E. coli* was stable and had a specific activity of 2.15 $\mu\text{mol}/\text{min}$ per mg. LC has proven to be a valuable reagent in high-throughput proteolytic assays^{13,14} and assays for screening inhibitors¹⁵ of its catalytic activity. Like BoNT/A (H₂), LC+belt and LC+H₂ induced neutralizing antibodies in mice. LC in phosphate buffer did elicit protective immunity in mice; however, LC-refolded and LC in acetate buffer did not. Because LC in acetate buffer had comparable activity to the LC in phosphate buffer, it was surprising that protective immunity in mice was not observed with the LC in acetate buffer. Further investigations will be required to decipher this result.

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TABLE 4. Protection and immunogenicity of LC, LC+belt, and LC+H₂ from *Escherichia coli*

| Construct | Inoculation dose (μg) | Geometric mean ELISA titer | Challenge dose (MLD ₅₀) | Survivors (survived/total) |
|-------------------|------------------------------------|-------------------------------|-------------------------------------|----------------------------|
| LC-refolded | 15 | 2.16 \times 10 ⁵ | 103 | 0/10 |
| LC-NaAcetate | 5 | 0.25 \times 10 ⁵ | 103 | 0/10 |
| | 15 | 0.31 \times 10 ⁵ | 104 | 0/10 |
| | | | 103 | 1/30 |
| | | | 104 | 0/30 |
| LC-phosphate | 15 | 1.04 \times 10 ⁵ | 103 | 17/19 |
| | | | 104 | 10/20 |
| LC+belt | 5 | 10.6 \times 10 ⁵ | 103 | 10/10 |
| | 15 | 6.06 \times 10 ⁵ | 103 | 10/10 |
| LC+H ₂ | 5 | 11.1 \times 10 ⁵ | 103 | 10/10 |
| | | | 104 | 10/10 |
| | 15 | 3.25 \times 10 ⁵ | 103 | 10/10 |
| | | | 104 | 10/10 |
| H ₂ | 5 | 0.17 \times 10 ⁵ | 104 | 10/10 |
| | 15 | 0.87 \times 10 ⁵ | 103 | 10/10 |
| Naive mice | 0 | 0 | 103 | 0/10 |
| | 0 | 0 | 103 | 0/10 |

LC, light chain; H₂, translocation domain; H₂, nontoxic fragment C; ELISA, enzyme-linked immunosorbent assay.

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Cell bank characterization and fermentation optimization for production of recombinant heavy chain C-terminal fragment of botulinum neurotoxin serotype E (rBoNTE(H_C): Antigen E) by *Pichia pastoris*

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Abstract

A process was developed for production of a candidate vaccine antigen, recombinant C-terminal heavy chain fragment of the botulinum neurotoxin serotype E, rBoNTE(H_C) in *Pichia pastoris*. *P. pastoris* strain GS115 was transformed with the rBoNTE(H_C) gene inserted into pHLD4 *Escherichia coli*—*P. pastoris* shuttle plasmid. The clone was characterized for genetic stability, copy number, and BoNTE(H_C) sequence. Expression of rBoNTE(H_C) from the Mut⁺ HIS4 clone was confirmed in the shake-flask, prior to developing a fed-batch fermentation process at 5 and 19 L scale. The fermentation process consists of a glycerol growth phase in batch and fed-batch mode using a defined medium followed by a glycerol/methanol transition phase for adaptation to growth on methanol and a methanol induction phase resulting in the production of rBoNTE(H_C). Specific growth rate, ratio of growth to induction phase, and time of induction were critical for optimal rBoNTE(H_C) production and minimal proteolytic degradation. A computer-controlled exponential growth model was used for process automation and off-gas analysis was used

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for process monitoring. The optimized process had an induction time of 9 h on methanol and produced up to 3 mg of rBoNTE(H₄) per gram wet cell mass as determined by HPLC and Western blot analysis.

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Keywords: Recombinant C-terminal heavy chain fragment of the botulinum neurotoxin serotype E; *Pichia pastoris*; Cell-bank characterization; Fed-batch fermentation; Potency studies

Nomenclature

| | |
|-----------------------|--|
| <i>D</i> | derivative factor |
| <i>F</i> | methanol feed rate (g/h) |
| <i>I</i> | integral constant |
| <i>m</i> | maintenance coefficient (g/g/h) |
| <i>P</i> | proportional constant |
| <i>K</i> | cell density correction factor |
| <i>T</i> | induction time |
| <i>V</i> | fermentation medium volume (l) |
| <i>X</i> | wet cell density (g/l) |
| <i>Y_{NH}</i> | observed yield of biomass to substrate (g/g) |

Greek letters

| | |
|------------|--|
| ϵ | error |
| μ | specific growth rate (h ⁻¹) |
| ν | specific methanol utilization rate (g/g/h) |

Subscripts

| | |
|----------|-------------------------|
| max | maximum |
| MeOH | methanol |
| <i>t</i> | at that particular time |
| 0 | at initial time |

1. Introduction

Botulinum neurotoxins, the most poisonous substances known to mankind, are finding increased attention due to their potential threat as a biological warfare agent (Medical and Public Health Management, 2001). The toxin produced by the bacteria, *Clostridium botulinum* and other closely related clostridial species, is a zinc endoprotease that acts to prevent the release of acetylcholine thus blocking neuromuscular transmission which, if untreated, progressively leads to skeletal muscle paralysis and eventually death from respiratory failure (Dreyer and Habermann, 1986; Simpson, 1986; Pellizzari et al., 1999). There are seven antigenically

distinct serotypes of the neurotoxin designated as A, B, C₁, D, E, F and G (Hatheway, 1989). These neurotoxins cleave specific sites on the soluble N-ethylmaleimide-sensitive factor-attachment protein receptor proteins or SNARE proteins (Schiavo et al., 1993; Foran et al., 1996; Niemann et al., 1994; Blasi et al., 1993). SNARE proteins are key components of the nerve cell system responsible for the release of the neurotransmitter acetylcholine into the synapse at the neuromuscular junction, which ultimately stimulates the associated muscle (Jahn and Südhof, 1999). The SNARE proteins consist of synaptobrevin on the vesicle membrane and syntaxin and synaptosome-associated protein of 25 kDa (SNAP25) at the synaptic membrane. Serotype E cleaves SNAP25 which prevents assembly of the synaptic fusion complex and therefore the fusion of the acetylcholine-containing vesicle and the synaptic membrane. This prevents the release of acetylcholine into the synapse resulting in a lack of stimulation of the downstream muscle fibers and results in muscle paralysis (Schiavo et al., 1993; Simpson, 1986; Byrne and Smith, 2000). Structurally, the botulinum neurotoxins have two domains, a 100 kDa heavy chain and a 50 kDa light chain bound together by a disulfide bond (DasGupta, 1989; DasGupta and Sugiyama, 1972). Functionally, the heavy chain consists of two subdomains, a domain at the N-terminus responsible for membrane transfer into the nerve cell, and a domain at the C-terminus responsible for binding to the nerve cell membrane. The light chain is zinc dependent proteases which cleave the SNARE proteins (Smith, 1998). However, both the non-toxic heavy and light chain fragments are antigenic and can elicit protective immunity in animals challenged with the toxin (Byrne and Smith, 2000). To counteract the threat from the lethal botulinum neurotoxin, various attempts were made to develop an effective vaccine against all serotypes. Initial attempts included development of a pentavalent (A–E) toxoid vaccine by the U.S. Army for immunizing Army personnel who might be exposed to biological

warfare. However, the toxoid vaccines poses several risks which include handling functional toxins, large volumes of formaldehyde and the current requirements for specific manufacturing facilities for growing spore-forming bacteria (Byrne and Smith, 2000). In addition, the toxoid vaccine candidates, which contain crude extract of inactivated *Clostridial* proteins, might influence immunogenicity of the vaccine (Byrne and Smith, 2000). Recombinant vaccines can be custom designed to be safe and effective. Proper choice of the vector can make the vaccine easy to produce and the culture easy to maintain, and thereby reduce production costs. Unwanted portions of the antigen that do not elicit protective immunity or pose health risks can be eliminated from the vaccine and is a huge advantage over the toxoids (Smith, 1998; Clare et al., 1991). The recombinant botulinum vaccine candidates were first expressed in *Escherichia coli*. However, large quantities of soluble antigen E could not be produced in this expression host due to formation of inclusion bodies which made refolding difficult resulting in a low yield (Smith et al., 2004).

Subsequently, a *Pichia pastoris* expression system was evaluated for expression of rBoNTB(H₂). Previously, *P. pastoris* was used to express high levels (27% of the total cell protein or about 12 g/L of culture) of tetanus toxin fragment C, a subunit vaccine candidate designed to provide protection against tetanus neurotoxin (Clare et al., 1991). *P. pastoris* is a commercially useful organism for high level expression of recombinant proteins with many advantages (Zhang et al., 2000; Gellissen, 2000). The organism grows on defined media to high cell densities on either glycerol or methanol as the sole carbon source (Zhang et al., 2000; Sinha et al., 2003) and heterologous protein production is under the control of a strong but tightly regulated alcohol oxidase promoter induced by methanol. *P. pastoris* can be grown to the desired cell density on glycerol as the carbon source and then on methanol for high level heterologous protein production (Cregg et al., 1987). In addition, expression can be controlled to direct expression of target proteins to either the intracellular compartment or to the extracellular medium by secretion. Expression studies with rBoNTB(H₂) found that secretion of rBoNTB(H₂) resulted in glycosylation due to *N*-glycosylation recognition sequences even though native botulinum neurotoxin is not glycosylated. Glycosylated rBoNTB(H₂) did not provide

protection in a mouse efficacy model while the unglycosylated rBoNTB(H₂) control provided the necessary protection (Byrne et al., 1998; Smith, 1998). The decision was made to express all rBoNTB(H₂) intracellularly to eliminate potential glycosylation. The expression of rBoNTB(H₂) is under the control of the alcohol oxidase promoter in a methanol utilization positive strain (Mut⁺), which is induced by methanol as the sole carbon source and repressed by other carbon sources like glycerol or glucose (Inan and Meagher, 2001).

The purpose of this work is to characterize a research cell bank suitable for Current Good Manufacturing Practice (CGMP) and to develop a fermentation process suitable for transfer to a CGMP facility for production of rBoNTB(H₂) for use as a vaccine candidate in clinical testing.

2. Materials and methods

2.1. Strain development

A rBoNTB(H₂) gene was synthesized based on the sequence of *C. botulinum* NCTC 11219 strain and *P. pastoris* codon usage (Loveless, 2001). The codon optimized rBoNTB(H₂) gene was inserted into the pHIL-D4 expression vector (Sreekrishna and Kropp, 1996) at the *EcoRI* site (Fig. 1). After amplification in *E. coli* DH5 α , the plasmid was linearized with *SrfI* and then transformed into *P. pastoris* GS115 (*his4*) by spheroplast procedure as described by Cregg and Kimberly (1998). Cells growing on minimal dextrose (MD) media lacking histidine were screened for copy number on YPD plates containing increased concentrations of antibiotic, geneticin (G418) up to 10 mg/mL. Cells grown in 25 mL of minimal glycerol medium without histidine (1.34% yeast nitrogen base (YNB), $4 \times 10^{-5}\%$ biotin, 1% glycerol/L sterile distilled water) to an OD_{600nm} of 4–8 were transferred to 2 L baffled flasks containing 175 mL of minimal methanol medium (1.34% YNB, $4 \times 10^{-5}\%$ biotin, 0.5% methanol) (Loveless, 2001). The cultures were harvested at 22.5 h by centrifugation at $2000 \times g$ for 5 min at 4°C. The cells were ruptured and the cell extract after centrifugation at $10,000 \times g$ was examined for best production by Western blot analysis. The best producing clone, *P. pastoris* [rBoNTB(H₂) E3] was selected as the production clone.



Fig. 1. The expression vector pHILd4/rBoNTE(Hc). The restriction enzyme site *EcoRI* is utilized for the insertion of the gene of the rBoNTE(Hc). The pHILd4 plasmid was derived by insertion of the gene encoding aminoglycoside 3'-phosphotransferase from pUC-4K into pHIL-D1 (Sreekrishna and Kropp, 1996).

2.2. Cell bank production

A single colony from a YPD plate was transferred to a test tube containing 10 mL of YPD medium. The test tube was incubated at 30 °C and 200 rpm in a rotary shaker for 24 h. Five millilitres of culture was used as inoculum for 100 mL of YPD media in 500 mL baffled shake flask. The culture was grown up to 8–10 OD_{600nm} using the same conditions described above. When the desired optical density was obtained, glycerol was added to a final concentration of 15% (v/v). The culture and glycerol were mixed thoroughly, and 1 mL of mixture was distributed aseptically into 2 mL cryovials (Sarstedt, Hayward, CA). The vials were stored in the vapor phase of liquid nitrogen.

2.3. Cell bank characterization experiments

2.3.1. Culture identity test

The culture identity test was performed by Accugenix Inc. (Newark, DE). In brief, a 500 bp region of the D2 segment of the 25–28S rRNA locus was amplified from purified DNA using the PE Biosystems' MicroSeq DS LSU rDNA fungal sequencing kit. Both DNA strands of the amplified fragment were sequenced using di-deoxy terminator sequenc-

ing chemistry and analyzed using ABI Prism 377 DNA sequencers. The data was assembled, aligned, and compared to a database of 1200 validated entries using the PE Biosystems' MicroSeq Microbial Analysis software.

2.3.2. Cellular morphology and cell viability

Cellular morphology was determined by the gram staining process. The cells were visualized with a microscope under oil immersion at 100× magnification to distinguish cell size and shape. Cell viability was obtained by counting colonies of cells grown on agar plates after suitable dilution of the original culture and reported as colony forming unit (cfu/mL).

2.3.3. Structural integrity of the inserted rBoNTE(Hc) gene

Structural integrity of rBoNTE(Hc) gene after cell bank manufacturing with *P. pastoris* rBoNTE(Hc) E3 clone was assessed by Southern blot analysis. Genomic DNA was isolated from *P. pastoris* rBoNTE(Hc) clone using MasterPure Yeast DNA Purification Kit (Epicentre, Madison, WI) from YPD grown culture. One microgram of genomic DNA was digested with *BstXI*, *EcoRI*, *EcoRV*, *HindIII*, *NheI* and *XbaI* and separated on a 0.8% Agarose gel. The DNA was transferred to a positively charged nylon membrane, Zeta-Probe GT (BioRad, Hercules, CA) using the method described by Southern (1975) and fixed to the membrane by a UV-Crosslinker. The membrane was pre-hybridized for 30 min at 40 °C with a hybridization solution supplied by DIG High Prime DNA Labeling and Detection Starter Kit II (Roche Diagnostics Corporation, Indianapolis, IN). Upon completion of pre-hybridization, the DIG labeled whole rBoNTE(Hc) gene in the same hybridization buffer was applied to the membrane as a probe. This hybridization step was performed for 16 h at 40 °C. Washing and detection protocol was carried out according to the manufacturer's instructions.

2.3.4. Insert copy number

Insert copy number was also estimated using Southern blot analysis. The chromosomal DNA of *P. pastoris* rBoNTE(Hc) E3 clone, GS115, and pHILd4/rBoNTE(Hc) plasmid DNA were digested with *XbaI* and run on a 0.8% TAE agarose gel. South-

ern blotting was performed according to the previously described method using the 1600 bp *NcoI/XbaI* fragment of *HIS4* gene encoded on the plasmid.

2.3.5. Insert DNA sequencing

The DNA sequence of the rBoNTE(H_2) gene inserted into the *P. pastoris* genome was determined as follows. The cells were induced for 12 h in methanol containing media (BMMY) before extracting total RNA. The cells were disrupted in a bead beater with TR1 Reagent® (Molecular Research Center Inc., Cincinnati, OH) and 0.5 mm silica zirconia beads with 7 cycles of 1 min each, equilibrated at room temperature for 5 min and then vortexed with chloroform. After incubation at room temperature for 10 min the resulting suspension was centrifuged and the aqueous phase was transferred to new microfuge tubes and extracted with isopropanol. These samples were centrifuged and the pellets of RNA were washed with 75% ethanol, centrifuged and air-dried. The pellets were re-suspended in FORMAZOL® (Molecular Research Center Inc., Cincinnati, OH) incubated for 10 min at 60°C in a multi-block heater and the RNA was stored at -80°C. Messenger RNA was purified from total RNA with Qiagen Oligotex mRNA Spin Column Purification Kit (Qiagen, Valencia, CA). The mRNA was used as template for one step RT-PCR using SuperScript One-Step RT-PCR for Long Templates kit from Invitrogen (Carlsbad, CA) using forward 5'-GAATTCACCATGGGAGAGAG-3' and reverse 5'-GAATTCCTATTATTTTCTTGCCATCC-3' primers.

The PCR product was ligated into pCRII-TOPO vector using TOPO TA Cloning Kit Dual Promoter from Invitrogen (Carlsbad, CA). Two positive clones were sequenced with a total of eight primers to ensure the sequence was covered twice.

2.4. Inoculum preparation

Frozen culture was thawed and added to previously sterilized BMGY medium (1% yeast extract, 2% (w/v) soytone, 0.1 M potassium phosphate buffer-pH 6.0, 1.3% (w/v) yeast nitrogen base and 1.2% (w/v) glycerol) in shake flasks. The culture was grown for approximately 24 h to an OD_{600nm} of 4–5. The seed culture (100 mL) was transferred aseptically to 2 L of the fermentation medium in 5 L Bioflo III/3000 fermentors or 500 mL seed culture was transferred aseptically

to 10 L of the fermentation medium in 22 L NLF22 fermentors.

2.5. Fermentation control

Bioflo III/3000 fermentors (5L) were interfaced with NBS BioCommand32 (New Brunswick Scientific (NBS) Company, Edison, NJ) software while NLF22 fermentors (22L) (Bioengineering AG) were interfaced with Batch Expert (Intelligent Laboratory Solutions, Inc., Naperville, IL) for complete supervisory control. The closed-loop feed control system consisted of a feed pump for methanol, balance for methanol, and a controller interface (Zhang et al., 2000). The NBS controller converted the feed rate to a pump setting which was then sent to the pump through the BioCommand32 hardware. The NLF Bioengineering fermentors were controlled by Mitsubishi FX programmable logic controllers (PLCs) which were interfaced with Batch Expert via an open connectivity (OPC) server and an OPC bridge. A dynamic data exchange (DDE) bridge was used to interface the Batch-Expert software with a VG Prima 8B mass spectrometer (Thermo Electron Corporation, Houston, TX), which was used for online analysis of residual methanol and other by-products, as well as determining the respiratory quotient. Open database connectivity (ODBC) bridge was also set up to exchange data between Batch-Expert and the database. The amount of methanol delivered was measured using a balance as the difference between the initial mass in the methanol tank and the current mass (Sinha et al., 2003). Both BioCommand32 and Batch Expert were set up to calculate the feed rates during glycerol and methanol feeding based upon the elapsed induction time and the amount of methanol actually delivered.

2.6. Fermentation conditions

P. pastoris cells were grown on basal salts medium which contained in g/L deionized water: KH_2PO_4 , 42.9; $(NH_4)_2SO_4$, 5.0; $CaSO_4 \cdot 2H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 11.7; K_2SO_4 , 14.3; glycerol, 20. In addition, 4.35 mL/L PTM1 salt was filter sterilized and added to the medium. PTM1 salts contained (in g/L deionized water): $CuSO_4 \cdot 5H_2O$, 2.0; $ZnCl_2$, 7.0; NaI, 0.08; $FeSO_4 \cdot 7H_2O$, 22.0; $MnSO_4 \cdot H_2O$, 3.0; Biotin, 0.2; $Na_2MoO_4 \cdot 2H_2O$, 0.2; boric acid, 0.02; $CoCl_2$, 0.5

along with H_2SO_4 , 2 mL. All chemicals were tested for composition and upon release by the Biological Process Development Facility, University of Nebraska-Lincoln (UNL-BPDF) Quality Assurance Unit were issued for use. All media components were entered in the media preparation logbook and copies were included in the UNL-BPDF's standard fermentation batch record, which are used for all research fermentations. Technology transfer batch records were used when the fermentation was scaled up to the 19 L. The batch record provides details of all phases of the fermentation with necessary checks, compliance with accepted ranges, and space to document any process deviations. Critical information also included culture information and seed bank lot number, manufacturer and lot number of all chemicals and supplies, and a detailed equipment list. The batch record referenced all pertinent standard operating procedures (SOP), which were also transferred to the contract manufacturing outsourcing (CMO) along with Material Safety Data Sheets (MSDS) and other safety precautions.

After inoculation of the fermentation medium, the cells were grown on glycerol (glycerol batch phase) until the glycerol was consumed, which was marked by a sudden and sharp increase in the dissolved oxygen level (a DO spike). This was followed by a glycerol fed-batch phase (linear feed: 13.3 g/L/h) to obtain a targeted cell density. A 63% (w/v) glycerol (13.3 g/L/h) feed containing 12 mL/L PTM1 salts was used as the carbon source during the glycerol fed-batch phase. At the end of the glycerol fed-batch phase, 2 g MeOH/L of broth was injected into the fermentor as a bolus to induce product gene expression. Simultaneously, the glycerol feed rate was programmed to decrease linearly from 13.3 g/L/h to zero over a 3-h period. This 3-h period is considered the transition phase as the cells adapt to methanol as the sole carbon source (Zhang et al., 2000). A pre-calibrated methanol sensor was used to monitor the level of methanol from off-gas which started decreasing after 1 h of the methanol addition and reached undetectable levels between 1.5 and 2 h after the addition of the bolus of methanol to the fermentor. At this time a continuous feed of methanol containing 12 mL/L of PTM1 salts (methanol fed-batch phase) was started and the cells were grown using an exponential methanol feed. Fermentations were performed at 30 °C and pH was controlled at 5.0 using saturated aqueous ammonium hydroxide throughout the fermenta-

tion. The dissolved oxygen (DO) was set at 40% of saturation and was controlled by a DO cascade of agitation (maximum of 800 rpm for 5 L fermentor and 1000 rpm for 22 L fermentor) followed by supplementing with pure oxygen to air sparging at 1 vvm. Samples were taken at regular intervals and analyzed for rBoNTE(H_4) by Western blot (qualitative) and HPLC (quantitative). A defined sampling schedule and sampling instructions were given in the batch record. The cells were harvested at the end of fermentation whenever necessary to support downstream processing and purification experiments.

2.7. Detection of rBoNTE(H_4) by Western blot

Fermentation samples were collected at various intervals and centrifuged at $8000 \times g$ for 10 min at 4 °C. The pellet was washed by re-suspending the pellet in cold lysis buffer (50 mM sodium phosphate, pH 7.5), centrifuged at 10,000 rcf for 5 min at 4 °C, decanting the supernatant, and re-suspending the pellet in the cold lysis buffer (10 mL buffer/g pellet) with 50 μL /g cell pellet) each of 0.5 M EDTA and 0.2 M phenylmethylsulfonylfluoride (PMSF). The cells were broken in a bead beater at 5 °C (3.7–3.9 g cold zirconia beads/g cell pellet) with 3 cycles of 1 min burst each with 5 min rest between cycles at 5 °C. The broken cells were centrifuged at $5000 \times g$ for 5 min at 5 °C to separate the cell extract from the cell debris and the zirconia beads and then re-centrifuged at $18,000 \times g$ for 10 min at 5 °C to remove any particulates prior to analysis. Protein bands from fermentation samples were separated on a 10% Bis-Tris gel with MOPS, pH 7.7 as the running buffer and then transferred to a (polyvinylidene difluoride) PVDF membrane using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). The PVDF membrane was soaked with blotto (5% (w/v) skimmed milk powder in Tris buffer saline) and treated sequentially with anti-BoNTE(H_4) antibodies derived from chickens for 1 h at a dilution of 1:3000, washed at least twice with Tris buffered saline (TBS) followed by treatment with peroxide labeled affinity purified goat anti-chicken secondary antibodies (Kirkland and Perry, Gaidnersburg, MA) at a dilution of 1:6667 for 1 h. The membrane was again washed several times with TBS and the image was developed using the ECL + plus Western Blotting Detection System (Amersham Biosciences, NJ). Purified rBoNTE(H_4) was used as the standard.

2.8. Estimation of rBoNTE(H_c) by HPLC

The concentration of rBoNTE(H_c) in the cell extract was estimated using a Waters (Milford, MA) high performance liquid chromatography system comprising a Model 600 four-solvent pump, Model 486 UV–vis detector and a Model 717 Plus auto-sampler. Waters HPLC software, Empower 5.0, was used for instrument control, data collection and data processing. Analysis was performed using a methyl acrylate copolymer (TSK gel phenyl 5-PW) hydrophobic interaction column (10 μ m, 7.5 mm \times 75 mm; Tosoh Bio-Science, Tokyo, Japan). The cell extract (0.5 mg protein per injection) was injected on to the column which was pre-conditioned with a mixture of 25% (v/v) of mobile phase A (0.2 M Tris–HCl, 2 mM EDTA, pH 7.7), 40% (v/v) of mobile phase B (1 M ammonium sulfate, 2 mM EDTA) and 35% (v/v) mobile phase C (2 mM EDTA) for 1 h. The rBoNTE(H_c) protein was eluted using a programmed gradient of the mobile phases A–C for 55 min at 1 mL/min. The composition of the individual solutions is the same as described earlier.

2.9. Protease assay

Protease activity in the cell extract was analyzed by measuring the fluorescent intensity of the liberated dye-labeled peptides from highly quenched fluorescent casein (Bodipy-casein FL) as a substrate (Jones et al., 1997). One unit of protease activity was defined as the unit increase in fluorescence intensity of Bodipy-casein FL as substrate with excitation at 485 nm and a fluorescence emission at 530 nm. Samples were analyzed using a SpectraMax M2 fluorescence spectrometer (Molecular Devices Corporation, Sunnyvale, CA) equipped with a 96 well micro plate reader.

2.10. Mouse potency bioassay

The potency of the purified rBoNTE(H_c) was determined using a mouse potency bioassay. A total of 7 groups of 10 mice each (Control: CD-1 mice, females, Charles River, Raleigh, NC) were intramuscularly vaccinated with 0.1 mL of diluted antigen. The antigen was diluted three-fold beginning at 8.1 μ g to 11 ng in 25 mM sodium succinate, 15 mM sodium phosphate, pH 5.0 with 5% mannitol and 0.2% Alhydrogel (HCl Biosector, Frederikssund, Denmark) as adjuvant. Twenty-one days following vaccination the mice were

challenged with 1000 mouse intraperitoneal LD₅₀ of botulinum type B toxin complex. Numbers of survivors were recorded 5 days post-challenge. Results were evaluated by the analysis of survival rates and calculation of the effective dose by probit analysis. Probit dose–response models were fitted to dose lethality data and the estimated parameters of the probit dose–response model were used to calculate ED₅₀ values, i.e., the theoretical effective dose of vaccine at which 50% of the animals vaccinated survive challenge. The 95% confidence interval for the ED₅₀ was calculated concurrently.

3. Results and discussion

3.1. Strain development and selection

The expression vector pPHLD4/rBoNTE(H_c) was constructed as shown in Fig. 1 (Loveless, 2001). The restriction enzyme site *EcoRI* was utilized for the insertion of the rBoNTE(H_c) gene fragment. The plasmid was linearized at the AOX1 promoter site with restriction enzyme, *SrfI* before transforming *P. pastoris* GS115 strain. Transformants were selected by expression of the histidinol dehydrogenase gene demonstrated by growth on histidine deficient regeneration medium (Cregg and Kimberly, 1998). Dose dependent resistance to the antibiotic geneticin (G418) was conferred by accumulation of the resistance determining enzyme aminoglycoside phosphotransferase (APT). His⁺ colonies which survived at 10 mg/mL G418 were screened for expression of rBoNTE(H_c) by methanol induction in shake flask culture. The best expressing clone was chosen for further studies by comparing on the basis of degree of band intensity (results not shown).

3.2. Cell bank characterization

Viable cell count of seed bank was 6.56×10^8 (cfu/mL) based on colony forming units (cfu) on Luria–Bertani (LB) plates by plating serial dilutions after freeze/thaw cycle of seed bank. Gram staining of the cells revealed that the cells were gram positive, ovoid cells with or without budding (results not shown) confirming the expected *P. pastoris* cell morphology. Cell growth on histidine-lacking media (MGY plates) also confirmed that GS115 host strain His⁺ pheno-

type was recovered by transforming with pHLD4 rBoNTE(H_c).

The rBoNTE(H_c) gene sequence was confirmed by sequencing of the RT-PCR product, which was the expected 1.3 kb fragment as described in Section 2. The consensus sequences of aligned DNA sequences with eight primers matched the theoretical rBoNTE(H_c) DNA sequence as well as the deduced amino acid sequence of rBoNTE(H_c) protein.

The copy number of the rBoNTE(H_c) gene inserted into the chromosome of *P. pastoris* rBoNTE(H_c)3E clones was estimated by Southern blot analysis (Fig. 2). The GS115 host strain resulted in a single band from the defective histidinol dehydrogenase (*his4*) gene when the genomic DNA was cut with *XbaI* enzyme and probed with a *NcoI/XbaI* fragment of the *HIS4* gene (Fig. 2, lane 3). The transformed host resulted in two bands, the 3 kb band corresponding to the chromosomal copy of *his4* gene, and an additional 10 kb band corresponding to a copy of *HIS4* gene from the expression vector (Fig. 2, lane 2). Copy number was estimated as a ratio of the intensity of the 10 kb band to the *his4* band (lane 2, 71/22 = 3.22). The experiment was repeated twice and the average copy number value obtained was 3.2. The copy number cannot be a fractional number, therefore, the copy number of rBoNTE(H_c) gene in *P. pastoris* (rBoNTE(H_c))E3 was estimated as three.

Structural integrity of rBoNTE(H_c) in *P. pastoris* rBoNTE(H_c))E3 clone was assessed by Southern blot analysis. This time the genomic DNA of the *P. pas-*

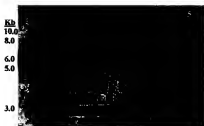


Fig. 2. Southern blot of GS115 host and *Pichia pastoris* rBoNTE(H_c))E3 strain using *HIS4* as a probe. Chromosomal DNAs were cut with *XbaI* enzyme. Lane 1, DNA ladder; lane 2, *P. pastoris* rBoNTE(H_c))E3 strain; lane 3, GS115 host strain; lane 4, pHLD4/rBoNTE(H_c)).

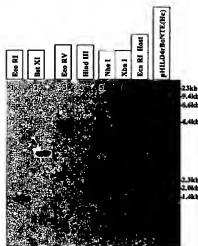


Fig. 3. Southern blot of GS115 host and *P. pastoris* rBoNTE(H_c))E3 strain using rBoNTE(H_c) as a probe. Lanes 1–6 are genomic DNA of *P. pastoris* rBoNTE(H_c))E3 cut with enzyme that are indicated as above the lanes; lane 7, genomic DNA of host strain GS115 cut with *EcoRI*; lane 8, pHLD4/rBoNTE(H_c)) plasmid DNA cut with *EcoRI*.

toris rBoNTE(H_c))E3 clone was digested by different enzymes and hybridized with DIG labeled whole rBoNTE(H_c) gene. The *EcoRI* digestion resulted in the expected 1.37 kb single band for transformed strain and no corresponding bands for the host strain (Fig. 3, lane 1). This band aligned as expected with 1.37 kb band of plasmid pHLD4/rBoNTE(H_c) digested with *EcoRI* restriction enzyme. Two bands were observed when genomic DNA was cut with *NheI* enzyme since the rBoNTE(H_c) gene contain an internal *NheI* site. *BstXI* digestion resulted in an expected 3 kb band (Fig. 3, lane 2) since *BstXI* digestion of the pHLD4/rBoNTE(H_c)) drops a 3 kb band from the plasmid by cutting outside of the rBoNTE(H_c) gene.

3.3. Shake flask growth kinetics and effect of inoculum age on fermentation

The *P. pastoris* rBoNTE(H_c))E3 clone was grown for 72 h under identical conditions in triplicate as described

in Section 2. The lag phase lasted 12 h, after which the cells grew exponentially at $\mu_{\text{observed}} = 0.1701$. The effect of inoculum age on fermentation productivity was evaluated. Inocula from shake flasks at the beginning of the exponential phase, middle of the exponential phase and the end of stationary phase, 22.75, 29.75 and 46.75 h, respectively, were used to inoculate three 5-L fermentors. Differences in the OD₆₀₀'s of the samples was compensated for by varying the volume used to inoculate the fermentor (OD₆₀₀ volume = 3286) so that the fermentor's initial OD₆₀₀'s were the same. The length of the batch phase with an inoculum age of 22.75 h was 19 h. The length of the batch phase increased to 22.25 h for both the 29.75 and 46.75 h inoculums. The age of the inoculum did not affect the final cell density of the batch phase indicating the yield coefficient of the cells was the same regardless of the age of the inoculum. Optimizing the inoculum age reduced total fermentation time by approximately 3 h.

3.4. Maximum methanol specific growth rate in 5-L fermentor

The maximum specific growth rate on methanol, $\mu_{\text{MeOH,max}}$ was determined by maintaining the residual methanol concentration in the fermentor below 2 g/L using a methanol sensor, which is below the inhibitory level (Zhang et al., 2000). A serial PID equation was developed to maintain the methanol set point below 2 g/L in the fermentor as described below:

$$F = P e + I \int e dt + D \times s dt \quad (1)$$

where F is the pump output in percent of maximum; e the error between sensor and set point; P the proportional factor; I the integral factor and D is the derivative factor.

Values of $P = 2$, $I = 0.01$ and $D = 30$ was found to be optimal for smooth control of methanol addition.

The $\mu_{\text{MeOH,max}}$ averaged 0.0567 h^{-1} in duplicate experiments, which is lower than 0.0709 h^{-1} for rBoNTA(H_c) (Zhang et al., 2000) and 0.08 h^{-1} for the wild type strain X-33 (GS115, His⁺) (unpublished results), indicating that there is a metabolic strain induced by production of rBoNTe(H_c). Recombinant BoNTe(H_c) analysis by HPLC revealed that cell growth at $\mu_{\text{MeOH,max}}$ resulted in a low yield of rBoNTe(H_c) (0.51 mg rBoNTe(H_c)/g WCW) and the



Fig. 4. Western blot of time profile of rBoNTe(H_c) production growing at μ_{max} ; lane 1, M – marker; lane 2, 0 h; lane 3, 1.9 h; lane 4, 22.4 h; lane 5, 29 h; lane 6, 34 h; lane 7, 45 h; lane 8, rBoNTe(H_c) standard. All times refer to elapsed induction time.

Western blot showed that rBoNTe(H_c) was substantially degraded at 22.4 h of methanol induction (Fig. 4). It has previously been observed that faster growth on methanol elicits higher protease accumulation in *P. pastoris* (Sinha et al., 2003, 2005) increasing the likelihood of proteolytic degradation of the product. Hence, growth rates below the maximum specific growth rates were investigated to determine an optimal growth rate for rBoNTe(H_c).

3.5. Optimal specific growth rate on methanol

Cells were grown on methanol at specific growth rates of 0.02, 0.03, 0.04 and 0.05 h^{-1} and the effect on rBoNTe(H_c) production was observed. The specific growth rates were controlled using a model feed equation described by Zhang et al. (2000):

$$F = (0.84\mu + 0.0071) K(X_0 V_0) e^{\mu t} \quad (2)$$

where F is the methanol feed rate (g/h); X_0 the wet cell density at the beginning of methanol feed (g/l); t the time of methanol fed-batch phase; V the fermentation medium volume (l) at the beginning of methanol feed and μ is the specific cell growth rate (h^{-1}).

Initially, a cell density correction factor of $K = 0.86$ was introduced to account for shrinkage and changes in wet cell density due to transition of carbon source from

glycerol to methanol, which lasted approximately the first 2–3 h of methanol feeding. Since this only lasts for 2–3 h, a value of $K=1$ was used. The observed growth rates closely matched the growth rates used in the growth model. Maximum rBoNTE(H₂) (1.93 mg/g WCW) was obtained when the cells were grown between $\mu=0.02$ and 0.03 h^{-1} . Subsequent experiments showed that optimum rBoNTE(H₂) production occurred at a specific growth rate of 0.0267 h^{-1} , which is the optimum for rBoNTE(H₂) (Zhang et al., 2000). Time course analysis of rBoNTE(H₂) samples by HPLC and Western blots showed that, irrespective of the specific growth rate on methanol, the maximum rBoNTE(H₂) was produced between 8 ± 3 and $22 \pm 3\text{ h}$ of induction approximately. After reaching maximum value at $22 \pm 3\text{ h}$ of induction approximately, rBoNTE(H₂) production decoupled from cell growth and rBoNTE(H₂) decreased steadily to undetectable levels after around 45 h of induction. The maximum yield of intact rBoNTE(H₂) was obtained at 9 h of induction.

Total amount of intracellular proteases were analyzed at different times during the methanol induction. It was observed that protease activity increased from the start of methanol induction and reached a maximum value at 9 h (following BoNTE(H₂) production) and remained at a constant level throughout the rest of the methanol induction phase (Fig. 5). The protease activity was found to have a direct correlation with anti-

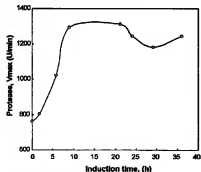


Fig. 5. Protease activities in cell extracts of time course samples from fermentation in 5 L bioreactor.

gen B production. It was determined that rBoNTE(H₂) purified from a 9 h induction was stable at $4-8^\circ\text{C}$ for 7 days as compared to rBoNTE(H₂) purified from a 27 h induction which degraded 5–10% based on SDS-PAGE under the same conditions (data not shown). It was this experiment that decided the 9 h methanol induction.

3.6. Extended glycerol feed rate and optimum MeOH induction time

The effect of growing *P. pastoris* cells to various high cell densities on glycerol prior to methanol induction was investigated. The objective was to determine the effect of extended glycerol feeding on protein expression and the optimal induction wet cell density for maximum product yield. The wet cell densities at the beginning of induction were varied from 200, 250, 300 and 350 g/L by growing the cells on a constant glycerol feed rate for an extended period of time. The cells were fed glycerol, as the sole carbon source, until they reached their desired wet cell weight (WCW) prior to a 9 h methanol induction. It was observed that the maximum specific yield of rBoNTE(H₂) per gram of wet cells was attained when cells were induced at a WCW of 200 g/L (Fig. 6). In comparison, induction at a WCW of 100 g/L resulted in the same BoNTE(H₂) specific yield as a WCW of 200 g/L. Induction at higher WCW resulted in a lower productivity per unit cell mass.

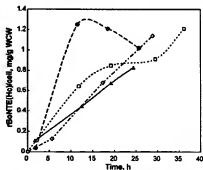


Fig. 6. Time course of rBoNTE(H₂) production after induction at different initial wet cell densities. (●) 200 g/L; (□) 250 g/L; (△) 300 g/L; (◇) 350 g/L.

3.7. Scale-up of fermentation

The rBoNTE(H₂) fermentation process was scaled-up to a 22 L Bioengineering NLF22 fermentor (Wald, Switzerland) to confirm product yield prior to transfer for CGMP production at the 100 L scale. A technology transfer batch record was implemented for the 22 L fermentation in preparation for transfer to a CGMP facility. In the batch record, the entire fermentation process was divided into several phases like inoculum batch up, inoculation, incubation, fermentor batch up, inoculation, batch, fed-batch and methanol induction phases, monitoring and sampling, harvest. Acceptable ranges were defined for each variable, e.g. pH: 5 ± 0.5 , temperature: $30 \pm 2^\circ\text{C}$, air flow: 1 ± 0.1 vvm flow, inoculum level: 50 ± 5 mL/L medium with a OD₆₀₀: 13 ± 2 . In the case of the methanol feed profile, the CMO did not have the ability program an exponential methanol feed rate so this was simulated by a series of linear steps, which overlapped the exponential profile. Using a series of linear steps produced the same quality and quantity of rBoNTE(H₂) and was easily transferred to the CMO. The technology transfer batch records provide more process specific information as compared to our CGMP batch records which are both process specific and equipment specific. The residual methanol level was monitored by off-gas measurement using a mass spectrometer interfaced to the control software Batch-Expert via a DDE bridge as described earlier. Methanol was detected in the fermentation broth when a bolus of methanol (1.5 g/L) was added for adaptation; the methanol level in the off-gas spikes as the cells do not utilize methanol immediately (the cells were utilizing glycerol as the carbon source), but with time the cells adapt to the methanol and this is reflected in a decrease of methanol in the off-gas (Fig. 7a). When the methanol level reduces to (50 ± 25 ppm), i.e. the cells utilize the initial bolus of methanol injected to the system, a second bolus of methanol (2 g/L broth) is introduced (as a bias) and the methanol feed started, so that the control system does not oscillate to control the methanol level at 2 g/L which is very critical for the process. The methanol level however was almost undetectable during induction, indicating that there was no accumulation of methanol in the medium (Fig. 7a). Analysis of off-gas data showed that the respiratory quotient (RQ) ranged from 0.5 to 0.7 and that the CO₂ evolution rate was 2–3% during methanol induction.

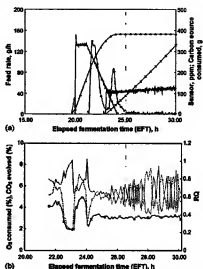


Fig. 7. Profile of various parameters and off-gas analysis during rBoNTE(H₂) fermentation in 19 L Bioengineering fermentor. (a) (—○—) Methanol feed rate; (—) glycerol feed rate; (▲) amount of glycerol fed; (■) amount of methanol fed; (□) methanol in off-gas. (b) (—○—) O₂ consumed; (■) carbon dioxide evolved; (—) respiratory quotient (RQ).

The fluctuations observed in oxygen uptake rate and RQ (Fig. 7b) were the result of a pulsating oxygen supply from the fermentor control unit which controls oxygen input by an on-off control at an oxygen requirement of less than 1 L/min. However, when the oxygen requirement is above 1 L/min, the oxygen supply was controlled at 0.1 L/min increments resulting in smooth oxygen supply. The respiration rate increased during the transition period and then decreased gradually to a steady value throughout induction. The observed specific growth rate was 0.0237 h^{-1} , which was close to the theoretical specific growth rate of 0.0267 h^{-1} . The induction WCW varied between 114 and 134.2 g/L in duplicate fermentation experiments. An induction time of 9 h produced 3.6 mg rBoNTE(H₂) per gram of wet cell. Cell growth, substrate utilization, and oxy-

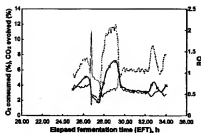


Fig. 8. Profile of off-gas analysis during rBoNTE(H_c) fermentation in 5L Bioflo fermentor: (---) Oxygen consumed; (•••) carbon dioxide evolved; (—) respiratory quotient (RQ).

gen consumption rate were comparable when the process was scaled-up from the 5 to 19 L scale, however, rBoNTE(H_c) yield was 1.25 mg/g WCW at 11.2 h in the 5 L bioreactor compared to 3.60 mg/g WCW produced at 9.05 h in the 19 L bioreactor. The cell density averaged an increase of 41.5 and 40 g/L at 5 and 19 L scale, respectively, during the methanol fed-batch phase. The yield coefficient on methanol was 0.8 g WCW/g MeOH consumed at the 5 L scale versus 0.71 g WCW/g MeOH consumed at 19 L scale. The RQ varied from 0.5 to 1.0 at the 5 L scale which was close to the RQ values of 0.5–0.7 at the 19 L scale (Figs. 8 and 9). However, the product quality was found to improve in the 19 L fermentor as no degradation fragments was detected by



Fig. 9. Western blot time profile of rBoNTE(H_c) production in 19L fermentor for large scale fermentation and purification. Lane 1, marker; lane 2, 0 h; lane 3, 2.3 h; lane 4, 9 h; lane 5, rBoNTE(H_c) standard. All times refer to elapsed induction time.

Western analysis compared to rBoNTE(H_c) produced in the 5L fermentors (Fig. 9). At this time there is no obvious or scientific reason to the significant increase in rBoNTE(H_c) yield at the 19 L scale, except that it was observed that 19 L system provided “smoother” control of fermentation variables and carbon source feeding.

3.8. Studies on mouse potency bioassay of rBoNTE(H_c)

The potency of the rBoNTE(H_c) produced from fermentations described above was determined using a mouse bioassay. Results were subjected to probit analysis to determine the ED₅₀, or theoretical antigen dose that will protect 50% of the mice from lethal injection. The survival of the mice after immunizations with rBoNTE(H_c) doses ranging from 11 ng to 8.1 µg was carried out. The calculated ED₅₀ for this potency assay was 214 ng, with 95% confidence limits ranging from 86 to 491 ng. The rBoNTE(H_c) for vaccination of mice was obtained from purification of protein from the 19 L scale fermentation.

4. Conclusion

A scalable fermentation process for the manufacture of rBoNTE(H_c) was developed in preparation for transfer to a CGMP manufacturing facility, using a fully characterized accession cell bank. An induction time of 9 h was optimal for minimizing proteolytic degradation of rBoNTE(H_c). The process is well-defined and scale-up studies at the 19 L scale indicate the ability to transfer the process to pilot-scale. This process is robust and should serve as a framework for development of the remaining botulinum recombinant vaccines under development, i.e. serotypes C, F, D and G.

Acknowledgements

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Evaluation of a botulinum fragment C-based ELISA for measuring the humoral immune response in primates

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Abstract

An enzyme-linked immunosorbent assay (ELISA) using botulinum neurotoxin serotype B recombinant fragment C (rBoNT-B(H_C)) was developed to measure specific humoral immune responses of monkeys vaccinated with a vaccine consisting of rBoNTB(H_C). Several fundamental parameters for a bioassay were evaluated. The evaluation results demonstrated that using BoNTB(H_C) as the capture antigen led to a specific and sensitive ELISA for botulinum type B antibody with excellent precision, accuracy, and linearity. There was a good correlation ($r=0.91$) between ELISA thers and neutralization bioassay thers. Experimental results suggested that the ELISA could be useful for detecting botulinum type B antibody levels and may supplement mouse neutralization bioassays during planned clinical manufacturing and clinical trials of rBoNTB(H_C) vaccine. Published by Elsevier Science Ltd on behalf of The International Association for Biologicals.

Keywords: Enzyme-linked immunosorbent assay; Botulinum; Recombinant vaccine; Assay validation

1. Introduction

Botulinum toxins are a group of seven neurotoxins produced by the bacterium, *Clostridium botulinum*. Botulinum toxins are extremely toxic to humans. Minute quantities ingested, inhaled, or absorbed through a break in the skin can cause profound intoxication and death. Use of botulinum toxins as a biowarfare or bioterrorism agent has long been a concern. In order to provide protection from the highly lethal organism and toxin, the development of vaccines has been a focus of the US Army's medical research laboratories [1–4]. The accurate and sensitive measurement of antibodies against botulinum toxins is an important concept in

vaccine development and clinical analysis. The currently accepted standard method to measure immune responses to botulinum neurotoxins is the traditional mouse lethality neutralization bioassay [5]. In this assay, serial dilutions of antiserum are mixed with a constant amount of toxin and then injected into mice. Protection from toxicity at a given antiserum dilution is the neutralizing antibody titer of the serum, which can be determined by the survival rate of the injected mice. This assay is extremely expensive, time consuming, and lacks precision and accuracy.

A number of alternate methods to mouse neutralization assay have been investigated for determination of both botulinum toxins and antibodies [6–13]. The enzyme-linked immunosorbent assay (ELISA) is, in most cases, an effective and suitable means to analyze immunological responses to antigens. The ELISA was developed to detect antibodies to botulinum toxins for

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investigation of botulism in cattle [14,15] and horses, [16] and to detect toxin in wild birds [17]. Proof of concept that an ELISA could detect botulinum antibody in human sera was first shown in infants with botulism in 1982 [18]. And most recently, an ELISA was evaluated for measuring the amount of neutralizing antibody against botulinum toxin type A in sera from human infants given botulinum immune globulin therapeutically (Buxton et al., personal communication).

Over the past few years, researchers at United States Army Medical Research Institute of Infectious Diseases (USAMRIID) successfully developed recombinant H_C fragment vaccine candidates for eliciting botulinum immunity [1]. These H_C -based vaccines will be evaluated in clinical trials as a potential, next generation vaccine for the US Army. As part of that effort, ELISA could be used alongside the mouse lethality neutralization assay and direct challenge of vaccinated animals to assess immune responses [1–4,19,20].

To support the future testing of the recombinant fragment C vaccines developed by the USAMRIID, we refined and optimized an ELISA to measure botulinum neurotoxin type B immune globulin G (IgG) by using botulinum neurotoxin serotype B fragment C (BoNTB- H_C) as the solid-phase antigen. A vaccination trial of monovalent BoNTB- H_C in nonhuman primates [21] (Boles et al., manuscript in preparation) allowed evaluation of several criteria of the proposed ELISA for its intended use. We measured IgG levels in sera of monkeys vaccinated with BoNTB- H_C by the ELISA. The data provide strong evidence of the utility of the botulinum H_C -based IgG ELISA, which may supplement the lethality neutralization assays performed during vaccine development and clinical trials.

2. Materials and methods

2.1. Capture antigens

Purified recombinant rBoNTB- H_C capture antigen was produced as previously described [3]. The protein concentration was 130 µg/ml, in 20 mM sodium phosphate, pH 7.5. The antigen solution was stored in a -70 °C freezer before use. The BoNTA- H_C [22] and the BoNTF- H_C [2] antigens were purified as previously reported. The BoNTC- H_C antigen was produced from *Pichia pastoris*, and purified by successive fractionation over Poros HQ 50, butyl sepharose 4 Fast Flow, and Fuso Haas phenyl 650m (manuscript in preparation). BoNTA toxin (List Biologicals, Campbell, CA), and BoNTB and BoNTF toxins (Food Research Institute, University of Wisconsin, Madison, WI), were commercially obtained and also used as capture antigens in this study.

2.2. Anti-botulinum type B monkey sera

Rhesus monkeys (*Macaca mulatta*), were divided into two sets of three groups: control group ($n=2$), low dose (1 µg vaccine) group ($n=6$) and high dose (5 µg vaccine) group ($n=6$). Each monkey in low- and high-dose groups was inoculated with rBoNTB- H_C (produced under current Good Manufacturing Practices (cGMP) at the Department of Biologics Research, Pilot Bioproduction Facility, Walter Reed Army Institute of Research, Silver Spring, MD) in 0.5 ml saline containing 0.2% Alhydrogel adjuvant, at weeks 0, 4, and 8. Monkeys in the control group received only saline/Alhydrogel. Sera were collected at intervals up to 2 years after the initial inoculation (Boles et al., manuscript in preparation).

2.3. Working calibration standards

Eight individual monkey sera from the high-dose group of the second set at 10, 12, 14 weeks after initial inoculation were pooled to make an internal reference standard for this assay. The botulinum type B IgG neutralization titers of sera were determined by a mouse neutralization bioassay [21]. The averaged type B IgG neutralization titer of the pooled serum was 3.5 IU/ml. Seven calibration standards were prepared with 5% skim milk buffer (skim milk powder, Difco, in phosphate-buffered saline with 0.05% Tween 20 and 0.9% benzyl alcohol, Sigma). The first standard (S1) was prepared from a 1:2000 dilution of the pooled serum. The S2–S7 were prepared as nine twofold serial dilutions of S1 before each assay. The final concentrations of type B IgG (in IU/ml) in the standard solutions are: S1=0.0018, S2=0.00088, S3=0.00044, S4=0.00022, S5=0.00011, S6=0.000055, and S7=0.000028.

2.4. ELISA

2.4.1. Plate layout

The ELISA was designed to measure BoNTB-specific IgG by using rBoNTB- H_C as the solid-phase antigen. The basic plate layout was designed to accommodate the testing of four test samples in triplicate at a proper starting dilution and followed four fourfold serial dilutions of the sample. Each plate included three triplicate positive controls, one triplicate negative control, seven triplicate calibration standards, and one triplicate no-template reagent control. All unknown samples and controls were prepared with 5% skim milk buffer.

2.4.2. ELISA procedure

The coating antigen rBoNTB- H_C was diluted to 2 µg/ml in PBS (pH 7.4, Sigma) and volumes (100 µl/well) were dispensed into PVC microtiter plates (Dynex Technologies, Chantilly, VA). After incubation at 4 °C overnight, the plates were washed three times with wash

buffer (PBS with 0.05% Tween 20, pH 7.4, Sigma) using an automatic ELISA plate washer (Ultrawash Plus, Dynex Technologies, Chantilly, VA). The plates were then blocked with 5% skim milk and incubated at 37 °C for 60 min. After washing three times, unknown samples (usually starting at 1:1000 dilution), standards, and controls were added to the plates (100 µl/well). The plates were incubated at 37 °C for 90 min and then washed. The secondary antibody, affinity-purified peroxidase-conjugated goat anti-human IgG (1 mg/ml, Kirkegaard and Perry Laboratories, Gaithersburg, MD), was diluted to 1:2000 with 5% skim milk buffer and added to plates (100 µl/well). The plates were incubated at 37 °C for 90 min and washed again. ABTS peroxidase substrate (two components, Kirkegaard and Perry Laboratories) was added (100 µl/well). The plates were incubated at room temperature for 25 min. The reaction was stopped by adding 100 µl/well of ABTS Peroxidase Stop Solution (5×, Kirkegaard and Perry Laboratories). The absorbance (OD) was measured in dual wavelength mode with wavelength 1=405 nm and wavelength 2 (reference)=490 nm, using REVELATION™ software program with a microplate reader (MRX, Dynex Technologies, Chantilly, VA). The mean OD, standard deviation (SD), and coefficient of variation (%CV) for each triplicate dilution of all standards, controls, and test samples were calculated automatically by the REVELATION™ program. The IgG concentration (IU/ml) of each unknown sample and control was calculated from each corresponding standard curve using a sigmoid regression equation (see Section 3). The REVELATION™ program determined the values automatically. For non-immune monkey sera, baseline absorbance was 0.027 ± 0.018 at 1:1000 dilution and 0.211 ± 0.051 at 1:100 dilution.

2.4.3. Inhibition study

A competitive inhibition study [23] was performed to assess specificity of the assay. Another pool of anti-botulinum type B monkey sera from the high-dose group of first set ($n=6$; average titer=2.8 IU/ml) was used for the study. Four different botulinum serotype H₂C proteins: BoNTB(H₂C), BoNTA(H₂C), BoNTF(H₂C), and BoNTC(H₂C), as well as BoNTB toxin, were used to inhibit the binding of anti-type B IgG in the pooled monkey serum to BoNTB(H₂C). The pooled monkey serum was diluted to 1:4000 with 5% skim milk buffer. Seven sixfold dilutions of the five inhibitors were prepared, respectively, starting at the highest concentration of 15 µg/ml to the lowest concentration of 0.00032 µg/ml with 5% skim milk buffer. An equal volume of diluted serum was added to each dilution of inhibitors and a tube containing buffer alone (as the 0% inhibition control). The neutralization titer of type B IgG in all mixed solutions was 0.00035 IU/ml. The final concentration of inhibitors in the mixed solutions was from 7.5 to

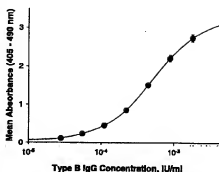


Fig. 1. A calibration curve of botulinum type B IgG in a pooled serum from BoNTB(H₂C)-vaccinated monkeys. The type B IgG concentrations of the standard solution ranged from 0.0018 IU/ml (S1, 1:2000 dilution of the pooled serum) to 0.000028 IU/ml (S7, 1:128,000 dilution of the pooled serum).

0.00016 µg/ml. The serum-antigen mixed solutions were placed at 4 °C while shaking overnight and were then analyzed by ELISA.

3. Results

3.1. Calibration curve

A calibration curve demonstrates the relationship between instrument response and known concentrations of the analyte [24]. For most immunoassays, the mean response was a nonlinear function of analyte concentration and is usually defined by a four-parameter logistic equation [25]

$$Y = D + (A - D) / [1 + (X/C)^B]$$

By using a pooled serum, in which type B IgG neutralization titer was 3.5 IU/ml, we established and optimized a calibration model with the four-parameter logistic equation. Data of the four parameters from 15 standard curves were calculated to be: $A = 0.04 \pm 0.04$; $B = 1.2 \pm 0.1$; $C = 6.1 \pm 1.5 \times 10^{-4}$ IU/ml; and $D = 3.4 \pm 0.2$, for this ELISA. The experimental results show that the four-parameter logistic equation fits the concentration-response very well. Fig. 1 shows a typical standard curve with extrapolated values, which was obtained from one of our experiments.

3.2. Specificity

To evaluate the specificity of the assay, a competitive inhibition study [23] was performed as described in

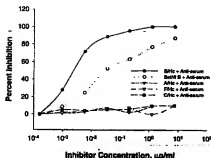


Fig. 2. Inhibition effects of different inhibitors on the specific binding of botulinum type B IgG in monkey serum to BoNTB(H_C) coating antigen. The type B IgG concentration in each serum-inhibitor mixed solution was 0.00035 IU/ml. The final concentration for each inhibitor was from 7.5 to 0.00016 µg/ml. The mean absorbance (OD) of the monkey serum in buffer was 1.5, which was used as 0% inhibition control.

Section 2. Fig. 2 shows the inhibition effects of BoNTB(H_C), BoNTB toxin, BoNTA(H_C), BoNTF(H_C), and BoNTC(H_C), on the antibody binding of botulinum type B immune monkey serum. As shown in Fig. 2, soluble BoNTB(H_C) completely inhibited binding at 1.2 µg/ml, and yielded 50% inhibition at 0.003 µg/ml, whereas the concentration of BoNTB toxin needed for 50% inhibition was 0.03 µg/ml. Other botulinum serotype H_C tested, such as A(H_C), F(H_C), and C(H_C), did not inhibit the type B antibody binding. These antibody-binding inhibition curves show that with BoNTB(H_C) as the capture antigen, the ELISA is specific and sensitive to botulinum type B antibody.

To further verify that type B IgG specifically binds only to BoNTB(H_C), not to other botulinum serotype antigens, we coated plates with different botulinum antigens, including BoTNB(H_C), BoNTB toxin, BoNTF(H_C), BoNTF toxin, BoNTA(H_C), BoNTA toxin, and BoNTC(H_C). A pooled positive type B monkey serum was measured in these plates by the ELISA. Fig. 3 shows that IgG in the sera from type B/H_C-vaccinated monkeys bound to botulinum type B(H_C) protein and toxin, but did not bind to H_C of types A, C, or F, or to types A and F botulinum toxins. This result agrees with the inhibition studies and indicates that the ELISA was highly specific for botulinum type B antibody from monkeys that received a monovalent botulinum vaccine candidate.

The parallelism of the test samples with calibration model was tested to verify the specificity of the ELISA [26]. Parallelism is a condition in which dilution of test sample does not result in biased measurements of the analyte concentration. Thus, when a test sample is

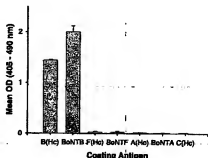


Fig. 3. The specific binding of botulinum type B IgG in monkey serum to various botulinum serotype coating antigens determined by ELISA. Type B IgG concentration in test solution was 0.00035 IU/ml. Coating antigen concentration was 4 µg/ml-well with 100 µl/well, in triplicate for each antigen.

serially diluted to result in a set of samples with analyte concentrations that fall within the quantitative range of the assay, there is no apparent trend toward increasing or decreasing estimates of analyte concentration over the range of dilutions. In other words, divergence of the plots indicates that the analyte in the test sample is different, or in a different form, from that in standard serum [27]. Concentration-response plots on dilutions of 12 individual positive botulinum type B monkey serum samples (all from 2 weeks after third vaccination) and calibration standards showed good parallel relationship (data not shown). The slopes of response-concentration curves for these test samples and standards are almost the same. The demonstration of parallelism indicates that the same analyte is being measured. The results suggest that the ELISA is specific and selective for botulinum type B IgG.

3.3. Precision

The precision within-run (well-to-well, from triplicate of same concentration and plate-to-plate, from five plates per day) for three positive quality control (QC) samples at different concentrations was evaluated. The %CVs of within-plate for high-, medium-, and low-concentration QC samples ranged from 1.3 to 11.7% for all five plates, except at one high concentration (%CV=17.5%). All within-day %CVs over five plates for the three QC samples were 10, 9.6, and 14.8% for high-, medium-, and low-concentration samples, respectively. Table 1 shows the within-run precision (from three plates per day) and day-to-day precision (from 18 plates run in 6 days) of the ELISA for three QC samples. The %CVs of within-run ranged from 1 to 5.9% over the 6 days. The day-to-day %CVs over 18 plates are 6.7, 3.8,

Table 1
Precision of the ELISA for botulinum type B IgG in QC samples of monkey sera*

| Simple | High | Medium | Low |
|-------------------------------|--|--|--|
| Nominal concentration (IU/ml) | 8.83×10^{-4} | 2.21×10^{-4} | 0.55×10^{-4} |
| Observed results | | | |
| | Concentration, $\times 10^{-4}$ IU/ml | Concentration, $\times 10^{-4}$ IU/ml | Concentration, $\times 10^{-4}$ IU/ml |
| Day 1 | 9.30 ± 0.44 | 2.23 ± 0.08 | 0.59 ± 0.02 |
| Day 2 | 9.49 ± 0.11 | 2.32 ± 0.07 | 0.59 ± 0.03 |
| Day 3 | 8.31 ± 0.42 | 2.15 ± 0.06 | 0.57 ± 0.03 |
| Day 4 | 8.78 ± 0.83 | 2.19 ± 0.09 | 0.56 ± 0.04 |
| Day 5 | 8.68 ± 0.50 | 2.22 ± 0.03 | 0.58 ± 0.01 |
| Day 6 | 8.42 ± 0.16 | 2.20 ± 0.10 | 0.59 ± 0.01 |
| Average concentration (IU/ml) | $8.83 \pm 0.60 \times 10^{-4}$ | $2.22 \pm 0.09 \times 10^{-4}$ | $0.58 \pm 0.03 \times 10^{-4}$ |
| %CV | 6.7 | 3.8 | 5.0 |

* Three plates were run in each day.

and 5.0% for high-, medium-, and low-QC samples. In addition, from 26 calibration curves, average %CVs of triplicate determinations for the seven standards (S1–S7) were 3.0 ± 2.5 , 3.4 ± 3.8 , 4.6 ± 4.2 , 5.4 ± 5.3 , 5.7 ± 5.4 , 5.0 ± 4.0 , and $11.2 \pm 12.1\%$, respectively.

3.4. Accuracy and linearity

Because there is no accepted standard of monkey botulinum anti-B(H_C) IgG for this assay, a pooled botulinum type B monkey serum as determined by a neutralization assay was used as a working reference value. A normal monkey serum was spiked with the pooled positive serum (type B IgG concentration: 3.53 IU/ml) at high (1.76 IU/ml), middle (0.20 IU/ml), and low (0.065 IU/ml) concentrations. The same sample was run in triplicate on each of three plates under same conditions in the same day. The observed concentrations from repeated plates were close to the nominal concentrations. The % recovery ranged from 93 to 118% and the %RE ranged from 5 to 18% for these samples.

3.5. Stability

To evaluate the stability of capture antigen BoNT-B(H_C) under freeze/thaw cycle conditions, six aliquots of BoNTB(H_C) antigen solution were separately treated by freezing/thawing at -70°C to room temperature for additional one to five times. The ELISA plates were then separately coated with the differently treated BoNT-B(H_C) antigen. A human IgG solution (type B IgG concentration: 4 IU/ml) derived from persons vaccinated with botulinum pentavalent was analyzed with these plates by the ELISA, starting at 1:1000 dilution and then 1:2 serial dilutions. Experimental results indicated that there was no discernable difference of the mean absorbances determined from differently treated capture antigen for the same antibody concentration, which means

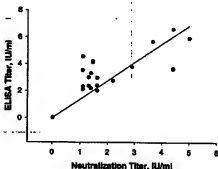


Fig. 4. Correlation of ELISA titer and neutralization titer of BoNTB(H_C)-vaccinated monkey sera bled at 10, 12, and 14 weeks after first immunization. $r=0.91$.

that BoNTB(H_C) is stable to these temperature and buffer conditions.

3.6. Correlation of ELISA titer and neutralization bioassay titer

We compared ELISA titer with protective antibody titers determined with a neutralization mouse bioassay (Boles et al., manuscript in preparation) of sera from monkeys after completion of vaccination (weeks 10, 12, and 14 after the first immunization) (Fig. 4). Statistical data showed that ELISA titers obtained with this assay were well correlated with the protective neutralization antibody titers. The correlation coefficient, r , was 0.91.

3.7. Application to routine analysis

Botulinum type B IgG levels in monkeys vaccinated as described above were measured over time with the

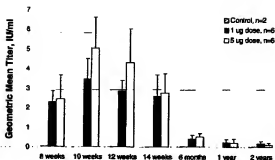


Fig. 5. ELISA geometric mean titers of sera from dosage groups of BoNTB(H_C)-vaccinated monkeys over the course of study.

ELISA. The ELISA IgG concentration was determined by comparing sample OD value with calibrated standard curves. Fig. 5 shows the geometric mean IgG titers of type B botulinum monkey sera from various time points over the course of study. Both 1 and 5 µg doses stimulated significant high antibody response. Geometric mean ELISA titer increased from baseline (pre-vaccination sera) to 2.30 IU/ml for the 1 µg dose group and to 2.45 IU/ml for the 5 µg dose group at 4 weeks after the second vaccination (8 weeks). As measured by ELISA, the specific IgG levels peaked at 2 weeks following the third injection (10 weeks). Six months after the first inoculation, the total anti-BoNTB(H_C) IgG levels measured by ELISA had declined.

4. Discussion

This ELISA was developed with the intent of supporting planned clinical trials of H_C-based recombinant vaccine products and was used here to measure the specific humoral immune response of monkeys vaccinated with rBoNTB(H_C) recombinant vaccine candidate. The capture antigen used for the ELISA was purified rBoNTB(H_C). Inhibition and specific binding studies found no cross-reactivity of anti-BoNTB(H_C) antibody with the purified BoNT(H_C) serotypes A, C, and F, as well as with types A and F toxins. Using BoNTB(H_C) as capture antigen, the ELISA was able to distinguish between serotype antibody levels. Parallelism of diluted type B monkey serum samples with diluted standard indicated that the matrix effect of monkey serum was not significant. Specific binding of type B IgG to B(H_C) and parallelism of sample dilutions with standard dilutions indicated that the ELISA was specific and sensitive to botulinum type B antibody. Using the four-parameter logistic equation to fit the mean absorbance to concentration, calibration curves used for the

ELISA includes 7 points, all of which were well fitted to the curve. The %CVs from triplicates of seven standards were very low. Experimental data showed that the lower limit of detection for diluted botulinum type B IgG was approximately 0.00011 IU/ml, which was more sensitive than the mouse bioassay. Experimental data demonstrate that the ELISA had excellent precision, accuracy, and dilutional linearity. The low %CVs from well-to-well, plate-to-plate, and day-to-day for QC samples and calibration standards indicate excellent precision of the ELISA. The closeness of observed type B IgG concentrations to their nominal concentrations in QC samples and spiked type B monkey serum samples demonstrated the accuracy and linearity of the ELISA.

The H_C of botulinum toxin may not be the only domain of the protein to induce protective antibodies after toxoid immunization. Light chain (LC) and the N-terminal domain of the heavy chain (H_N) elicit protective immunity when used to immunize rabbits [28] or mice [29]. The ELISA does detect anti-botulinum IgG in sera from individuals vaccinated with toxoid vaccine. In our stability experiment, the ELISA was applied to measure the type B IgG concentration in a human IgG preparation from sera of volunteers vaccinated with botulinum pentavalent toxoid vaccine. Results suggest that the H_C-based ELISA could also provide useful information for the detection of antibodies induced by whole toxoid vaccine.

ELISA measures total neutralizing and non-neutralizing antibodies binding to a given antigen, but does not specifically measure the neutralizing antibody to the toxin. In this study, we assessed the analytical performance of the ELISA. Comparison of the ELISA titers with neutralization titers of monkey sera from 10, 12, and 14 weeks after first vaccination showed strong evidence that there is a good correlation between these two methods ($r=0.91$). Previous reports also showed positive correlations between these two assays. For

example, in a phase I clinical trial [30], in which purified type F toxoid was administered to volunteers, serum samples were evaluated for an antibody response at various time intervals over 1 year by mouse bioassay and ELISA. After completion of the primary vaccination series, ELISA titers correlated well with mouse neutralization bioassay titers ($r=0.86$). An earlier study showed that the correlation coefficients between ELISA and neutralization titer was $r=0.69$, $P<0.0001$ for 186 pentavalent (ABCDE) toxoid vaccinated human sera for type A immune response and $r=0.77$, $P<0.0001$ for 168 sera for type B antibody [31].

We believe, if there exists a high correlation between protection and serum titer, then the ELISA titers can be used as an alternative to actively challenging animals with botulinum toxins. Several studies using whole neurotoxin as capture antigen, demonstrated that ELISA can predict protective immunity. For botulinum type F(H₂C)-vaccinated animals, all mice with ELISA titer of 1:100 or greater survived lethal toxin challenge [2]. For botulinum type A(H₂C)-vaccinated mice, ELISA titers correlated well with survival and neutralization titers: 82 out of 83 mice with titers ≥ 1600 survived toxin challenge [19]. Vaccinating mice with a DNA-vaccine containing the H₂C gene fragment provided protective immunity with a positive relationship between the level of protection and the level of ELISA reactive antibody [20]. In rhesus monkeys vaccinated with type B(H₂C), ELISA titers of sera after completion of the primary series correspond to neutralization titers and are predictive of survival against aerosol challenge [21]. These studies show that ELISA offers great potential as a surrogate of protective antibody levels in sera from volunteers vaccinated with H₂C vaccine candidates. Recombinant (H₂C)-based ELISA should provide better indications of protective immunity after vaccination with BoNT(H₂C) vaccine candidates than holotoxin-based ELISA.

Botulinum neurotoxins share many homologies to tetanus neurotoxin, therefore, results achievable with tetanus IgG ELISA titers may be possible with botulinum immune diagnostics. As early as 1983, studies indicated a good correlation between tetanus IgG ELISA titers and neutralization titers [32]. A collaboration study [33] carried out in Europe for pooled serum samples from guinea pigs vaccinated with tetanus toxoid vaccines observed excellent coefficients of correlation between ELISA and toxin neutralization test ($r=0.986$, 0.925 and 0.977, respectively, from three laboratories), as well as between ELISA and the toxin binding inhibition test ($r=0.92$). And the correlation between ELISA and the challenge test achieved a 93% predictive value. Tetanus toxin immunity has been monitored in human populations with commercial ELISA kits and ELISA technique in the US, as well as in many other countries [34–37]. Tetanus toxin fragment C (TTFC) has been

used as coating antigen to determine TTFC-specific antibodies in mouse sera and bronchoalveolar lavage fluids by ELISA [38].

In conclusion, by using BoNTB(H₂C) as capture antigen, we showed that the ELISA specifically and sensitively measured botulinum type B antibody levels. The assay had good precision, accuracy, and linearity. ELISA titers were well correlated with the protective neutralization bioassay titers. The ELISA could be useful for detecting botulinum type B antibody levels and could supplement the lethality neutralization assay during vaccine development and clinical trials.

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Evaluation of the Therapeutic Usefulness of Botulinum Neurotoxin B, C1, E, and F Compared with the Long Lasting Type A

BASIS FOR DISTINCT DURATIONS OF INHIBITION OF EXOCYTOSIS IN CENTRAL NEURONS*

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Seven types (A–G) of botulinum neurotoxin (BoNT) target peripheral cholinergic neurons where they selectively proteolyze SNAP-25 (BoNT/A, BoNT/C1, and BoNT/E), syntaxin (BoNT/C1), and synaptobrevin (BoNT/B, BoNT/D, BoNT/F, and BoNT/G), SNARE proteins responsible for transmitter release, to cause neuromuscular paralysis but of different durations. BoNT/A paralysis lasts longest (4–6 months) in humans, hence its widespread clinical use for the treatment of dystonias. Molecular mechanisms underlying these distinct inhibitory patterns were deciphered in rat cerebellar neurons by quantifying the half-life of the effect of each toxin, the speed of replenishment of their substrates, and the degradation of the cleaved products, experiments not readily feasible at motor nerve endings. Correlation of target cleavage with blockade of transmitter release yielded half-lives of inhibition for BoNT/A, BoNT/C1, BoNT/B, BoNT/D, and BoNT/E (>31, >25, ~10, ~2, and ~0.6 days, respectively), equivalent to the neuromuscular paralysis times found in mice, with recovery of release coinciding with reappearance of the intact SNAREs. A limiting factor for the short neuromuscular durations of BoNT/F and BoNT/E is the replenishment of synaptobrevin or SNAP-25, whereas pulse labeling revealed that extended inhibition by BoNT/A, BoNT/B, or BoNT/C1 results from longevity of each protease. These novel findings could aid development of new toxin therapies for patients resistant to BoNT/A and effective treatments for human botulism.

Seven immunologically distinct serotypes of botulinum neurotoxin (BoNT)¹ (A–G) from *Clostridium botulinum* are homologous proteins consisting of a heavy and light chain linked by an essential disulfide and noncovalent interactions that specifi-

cally block the release of acetylcholine at the neuromuscular junction (reviewed in Refs. 1–3). BoNTs cause botulism, the majority of human outbreaks being caused by types A, B, or E (1); however, they are remarkably useful as therapeutic agents (see below). The striking potency of the toxins and their cholinergic selectivity arise from their multiple domains mediating: (i) targeting to motor nerve endings via high affinity interaction with ecto-acceptors located exclusively thereon (4, 6) and (ii) endocytosis (5) followed by translocation of a LC-containing moiety into the cytosol. Their LCs are Zn²⁺-dependent endoproteases that selectively cleave single peptide bonds (except for BoNT/C1; see below) in one of three SNARE proteins that constitute the components of a ternary complex responsible for vesicle docking/fusion during regulated exocytosis (7). Synaptosomal-associated protein of 25 kDa (SNAP-25) (8) is proteolyzed by BoNT/A, BoNT/C1, and BoNT/E at separate sites near the C terminus: Gln¹⁹⁷–Arg¹⁹⁸, Arg¹⁸⁸–Ala¹⁸⁹, and Arg¹⁸⁸–Leu¹⁸¹, respectively (3). Another plasma-membrane protein, syntaxin (Stx1) (reviewed in Ref. 9), is also cleaved by BoNT/C1, and synaptobrevin, a synaptic vesicle protein (Sbr) (10, 11) is cleaved by BoNT/B, BoNT/D, BoNT/F, and BoNT/G, and tetanus toxin (TeTx). BoNT/A- or BoNT/E-truncated SNAP-25 (termed SNAP-25_A or SNAP-25_E, respectively) remains membrane-bound, but release is inhibited; in the case of SNAP-25_A, some assembly and disassembly of the ternary complex can still occur (12, 13). Truncation of Stx1 or Sbr by the requisite BoNT results in detachment of their cytosolic domains.

When applied locally to humans for the treatment of dystonias (reviewed in Ref. 14), BoNT/A, BoNT/B, and BoNT/E cause neuromuscular paralysis for more than 4 months, ~2 months, or <4 weeks, respectively (15, 16); the limited results available for type C1 suggest a duration less than or equal to that of BoNT/A (17). It is unclear why the recovery times in rodents are shorter and yet show the same rank order (1–2 months (BoNT/A), 21 days (BoNT/B), 7 days (BoNT/F), and 4 days (BoNT/E)) (18, 19).² Insight has been gained into the sequence of events involved in the protracted resumption of neurotransmission in BoNT/A-poisoned motor endplates by monitoring synaptic function in individually identified nerve endings of living mice (18). This showed that the transient appearance of functional nerve sprouts mediates a partial return of neuromuscular function, with full recovery relying on the originally affected endings regaining the ability to mediate chemical transmission. In chromaffin cells, the persistence of BoNT/A

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¹ The abbreviations used are: BoNT, botulinum neurotoxin; DIV, days *in vitro*; ERK, Erbb2-Kinase-HER2; LC, light chain; Sbr, synaptobrevin; SNAP-25, 25-kDa synaptosomal-associated protein; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptor; Stx1, syntaxin; TeTx, tetanus toxin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

² Meunier, F. A., Lisk, G., Seear, D., and Dolly, J. O. (2003) *Mol. Cell. Neurobiol.*, in press.

proteases for many weeks contributes to the extended inhibition of secretion; also, SNAP-25_A has been shown to be inhibitory (21–23). Of particular interest, Elosegui *et al.* (16) reported that co-treating human endplates with BoNT/A and BoNT/E results in a more rapid recovery of neuromuscular function, equivalent to that of BoNT/E alone, prompting many scientists (18, 22, 24) to suggest that both proteases have equivalent lifetimes in the motor nerve ending and the prolonged paralysis by BoNT/A arises from slow replacement of SNAP-25_A. Accordingly, Meunier *et al.* observed that type E hastens the removal of inhibitory SNAP-25_A from BoNT/A-treated mouse neuromuscular synapses by converting it to SNAP-25_E, which is replaced rapidly; thus, resumption of synaptic transmission is accelerated.

In this study, biochemical analyses (not practical with motor nerve endings or isolated motoneurons; see "Discussion") were performed on cultured cerebellar neurons to quantify the half-lives of toxin inhibition and the rates of turnover of SNAREs and their toxin-cleaved products. Although noncholinergic, these neurons provide a useful model for studying the intracellular fates of BoNTs, because we observed the same relative durations of neuromuscular actions of BoNT/A, BoNT/B, BoNT/C1, BoNT/E, and BoNT/F as measured in motor nerves *in vivo* (see above). In addition, these homogeneous cerebellar neurons are very susceptible to BoNTs and could be obtained in sufficient numbers for these quantitative measurements. In this way, we have extended earlier findings (22, 25) and explained how exocytosis can be blocked for dissimilar periods by the different BoNT serotypes.

EXPERIMENTAL PROCEDURES

Materials.—Cell culture media and general reagents were supplied by Sigma-Aldrich. N2 supplement and serum (extensively dialyzed before use) were from Invitrogen. [¹⁴C]glutamate was from Amersham Biochemicals, and [³⁵S]methionine was from ICN. Monoclonal antibodies selectively reactive with STx1, Shv2, or SNAP-25 were purchased from Sigma-Aldrich (clone HPC-1), Syntex Systems (clone 69.1), and Sternberger Monoclonals Inc. (clone SMD-61), respectively. IgG specific for SNAP-25 (C-terminal residues 195–206), Shv1 and 2 (62-residue peptide residues 32–94 of human Shv2; termed HV62), synaptotagmin II (last 20 amino acids), and SNAP-23 (11 residues at the C terminus) were produced in rabbits and affinity-purified as before (26, 27). Anti-glial fibrillary acidic protein IgG was a gift from Dr. G. P. Wilkin. All of the neurotoxins used were of >96% purity, fully checked as assessed by SDS-PAGE and protein staining, and exhibited maximal lethalities *in vivo*. Pure BoNT/F M complex (BoNT/F7) was supplied by Wako Chemicals (Osaka, Japan). All work with BoNTs was performed using approved, strictly enforced safety precautions.

Preparation and Maintenance of Cerebellar Granule Neurons: Exposure to BoNTs and Assay of Glutamate Release.—These cells were dissociated from the cerebella of 7–8-day-old rats (28) and suspended at $\sim 1 \times 10^6$ /ml in 3 parts of basal Eagle's medium and 1 part of 40 mM HEPES-NaOH, pH 7.3, 7.8 mM KCl, 37.6 mM D-glucose, 2.8 mM NaClO₄, 1.6 mM MgSO₄, and 1.0 mM NaH₂PO₄, as well as 1x N2 supplement, 1 mM L-glutamine, 60 units/ml penicillin, 60 µg/ml streptomycin, and 3% (v/v) horse dialyzed serum. An aliquot (1 ml) of this cell suspension was added to each of 16-mm-diameter poly-D-lysine coated well (i.e., 24-format) and cytosine-β-D-arabino-furanoside (40 µM) added after culturing for 20–24 h in 5% (v/v) CO₂; the neurons were maintained by replacement every 10 days with the same freshly prepared medium. This preparation is reported to contain largely (90–95%) glutamatergic interneurons (29), up to 8% γ-aminobutyric acid-ergic cells and 3% glial fibrillary acidic protein-containing astrocytes (30), controlled using an anti-sialic acid agent. Where specified, the neurons were exposed to toxin (0.2–µm filter sterilized) in culture medium for 24 h; unbound toxin was removed by three washes (over 10 min) with Krebs-Ringer-HEPES (KRH; 20 mM HEPES-NaOH, pH 7.4, 128 mM NaCl, 5 mM KCl, 1 mM NaH₂PO₄, 1.4 mM CaCl₂, 1.2 mM MgSO₄, 10 mM D-glucose, and 0.06 mM mg/ml bovine serum albumin, pH 7.4), and then the culture medium was replaced.

For measurement of transmitter release, the neurons were washed four times with O₂-pregassed KRH and incubated with 0.4 ml of the latter buffer containing 0.25 µCi/ml [¹⁴C]glutamate (i.e., a glutamate

precursor) (31); all of the steps were performed at 37 °C. After 45 min, the neurons were washed thrice briefly and twice for 5 min each time prior to a 5-min incubation in KRH containing 1.4 mM Ca²⁺ or 0.5 mM EGTA (i.e., to assess Ca²⁺-dependent, resting release); the aliquots were retained for measurement of [¹⁴C]glutamate content by ion exchange HPLC analysis and scintillation counting (31). A modified KRH buffer containing 50 mM KCl (with a reduced NaCl content of 83 mM to maintain osmolality) and 1.4 mM Ca²⁺ or 0.5 mM EGTA was added for a 5-min stimulation period. The amounts of Ca²⁺-dependent [¹⁴C]glutamate released into basal and K⁺-stimulated samples were measured as above, expressed as percentages of the total cell content, and the evoked component was calculated. Glia from rat cerebella were cultured in medium lacking an anti-sialic acid agent and containing 10% (v/v) fetal calf serum (nonaltered); 2 days before use, 0.1 mM glutamate and glycine were added to kill any residual neurons (32).

The use of extensive washing steps removes stick or dead cells that detach from wells, precluding a significant contribution to the experimental measurements. The healthy state of the cells remaining bound is indicated by several important criteria, including their abilities to efficiently perform multiple energy-dependent steps and attain up to 90% proteolysis of SNAREs by very low BoNT concentrations.

Pulse Labeling and SNARE Immunoprecipitation.—Neurons $\sim 5 \times 10^6$ /well (35-mm diameter) were washed four times with O₂-pregassed KRH and incubated in a modified culture medium retaining all of the above-noted additives except lacking serum and L-methionine but instead containing [³⁵S]methionine (50–100 µCi/ml). After 4 h, the neurons were washed twice and harvested immediately or "chased" in conventional medium supplemented with 0.25 mM unlabeled L-methionine. Washed neurons were detergent solubilized for 30 min (0.1 ml) using 2% (w/v) CHAPS and 2% (w/v) n-octyl β-D-glucopyranoside in 20 mM HEPES-NaOH, pH 7.4, containing 10 mM EDTA, 150 mM NaCl, 1% (v/v) bovine serum albumin, and 2% (w/v) of a protease inhibitor mixture (P6346; Sigma). All of the steps were performed at 0–4 °C. Insoluble material was removed by centrifugation at 15,000 × g for 40 min, and the extracts were incubated for 3–4 h in an end-over agitator with the relevant anti-SNARE Ig-protein A-agarose complex (10 µg of IgG/0.1 µl of resin). Resin was collected by centrifugation (5 × 100 × g) and washed eight times over 90 min (1 ml each) with solubilization buffer lacking the protease mixture and containing only 0.1% (v/v) each of CHAPS and n-octyl β-D-glucopyranoside. Neurotransmitter SDS-PAGE sample buffer was added to the agarose slurry and heated at 80 °C for 30 min. The radioactive immunoprecipitated SNAREs were subjected to SDS-PAGE, fixed, treated with Amplify™ (Amersham Biochemicals), dried for fluorography, and detected using Hyperfilm MP™. Control experiments found that BoNT/A, BoNT/B, BoNT/E, or BoNT/F treatments had no effect on protein synthesis, by measuring the amounts of radioactivity incorporated into precipitable protein relative to toxin-free controls (measured by scintillation counting; data not shown).

Immunoblotting and Quantitation of Antigens.—Immediately after assaying transmitter release, the cells were solubilized in 1% (v/v) SDS in 20 mM HEPES-NaOH, pH 8.5, containing 20 mM EDTA plus 150 mM NaCl; the total protein was quantitatively isolated using dodecylmethanol precipitation (outlined in Ref. 27). For optimal resolution of intact SNAP-25 from its toxin-truncated products, the samples were subjected to SDS-PAGE using NOVEX™ 12% Bis-Tris gels and a MOPS-based buffer system (Invitrogen). The proteins were electrotransferred and immunoblotted, as detailed previously (27), with detection by anti-species-specific IgG conjugated to horseradish peroxidase and visualization by enhanced chemiluminescence. The blots were densitometrically scanned, and the bands were quantified using image analysis software (Scion Image for Windows); the standard curves of the amounts of SNARE plotted against band intensity were constructed to allow accurate quantitation.

RESULTS

Recovery of Neuroexocytosis from BoNT/E- or BoNT/F-Inhibited Rat Cerebellar Neurons Is Rapid and Coincident with the Respective Reappearance of Intact SNAP-25 or Shv2.—Initially, the central neurons were chosen to be suitable for studying the dynamics of SNAREs and neuroexocytosis. Cerebellar granule cells, maintained under partial depolarization (31, 33), developed over time into mature neurons, establishing numerous neurite contacts (Fig. 1A) that are known to form functional synapses (34, 35). Immunoblotting revealed a minimal content of glial fibrillary acidic protein-reactive astrocytes but an abundance of STx1, Shv2, SNAP-25, and synaptotagmin 1, being

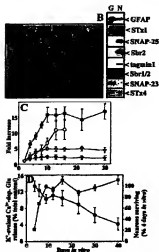


Fig. 1. Elevation of Ca^{2+} -dependent transmitter release and concomitant increase in SNAP-25 contents during development of cerebellar neurons lack of SNAP-43. *A*, the rat neurons (prepared as detailed under "Experimental Procedures") were cultured for 14 DIV before visualization by interference contrast microscopy. *B* and *C*, equal amounts (10 μg) of protein from neurons (*N*) and glia (*G*) (see "Experimental Procedures") were subjected to immunoblotting with the antibodies specified; primary IgG were detected using horseradish peroxidase-labeled secondary IgG and visualized by enhanced chemiluminescence. Sbr isoform 2 was specifically detected with a monoclonal 69.1 while both isoforms (*Sbr1/2*) were visualized with anti-KV262 (see "Experimental Procedures"). The temporal expression of STx1 (*V*), SNAP-25 (*V*), *Sbr2* (\bullet), or synaptotagmin (\square) was quantified by densitometric scanning of blots, and the fold increase in the immunoreactivity of each was expressed relative to the signals present at 2 DIV (*C*, only). *D*, The cells were loaded with ^{45}C -labeled glutamine for the quantitation of Ca^{2+} -dependent evoked release of glutamate (\bullet) as outlined in "Experimental Procedures" and assessment of neuron survival by microscopy (\circ). The data are the means \pm S.D. ($n = 3$ or 4).

much more prominent in neurons than glia (Fig. 1*B*). In contrast, SNAP-23, a BoNT/A-insensitive but BoNT/E-cleavable non-neuronal SNAP-25 homolog (27, 36, 37) was apparently absent from the granule cells but present in glia (Fig. 1*B*). Interestingly, the expression of STx1 and, particularly, SNAP-25, *Sbr2*, and synaptotagmin1 increased markedly during neuron development and synaptogenesis (Fig. 1*C*) concomitant with the maturation of the evoked exocytotic response, reaching a plateau at 10–13 days in culture (Fig. 1*D*). Importantly, for the purpose of studying SNARE function and the persistence of BoNT action, sufficient quantities of the developed neurons remained viable for several weeks (Fig. 1*D*), allowing the dynamics of SNARE expression and turnover to be investigated (see below).

Dose dependences for inhibition of evoked exocytosis were measured in granule cells exposed to BoNT/E or BoNT/F for 24 h; a maximum inhibition of ~80% and ~90% was seen, with corresponding losses of intact SNAP-25 (BoNT/E; Fig. 2, *A* and *B*) or *Sbr2* (BoNT/F; Fig. 2, *C* and *D*). The different concentrations of the two toxins necessary to give 50% blockade of evoked release, 43 pM and 1.35 nM respectively, occur with their disparate specific neurotoxicities in mice (data not shown). After BoNT/E removal by washing, the extent of the initial inhibition (i.e. day 0) and the amounts of SNAP-25_i decayed

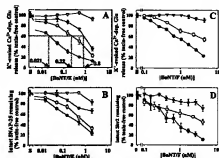


Fig. 2. BoNT/E and BoNT/F cause short-lived blockade of transmitter release that coincides with the reappearance of intact SNAP-25. *A*, after incubation of cerebellar neurons (7 DIV) for 24 h at 37°C in culture medium containing BoNT/E (*A* and *B*) or BoNT/F (*C* and *D*), the washed cells were assayed immediately at day 0 (\bullet), or the medium was replaced and the culture was maintained for 2 (\square), 4 (∇), or 7 (\triangledown) days following BoNT/E. *A* and *C*, evoked transmitter release was quantified (means \pm S.D.; $n = 3$ or 4). *B* and *D*, equal amounts of proteins were immunoblotted using BML-61 Ig (SNAP-25) and anti-KV262 Ig (*Sbr2*) to assess the extent of SNARE proteolysis (means \pm S.D.; $n = 3$), calculated after densitometric scanning of the blots. The extrapolated toxin concentrations causing equivalent blockade of transmitter release at various recovery times were used to calculate the $t_{1/2}$ of inhibition (example shown in *A*).

progressively over 2–7 days (Fig. 2, *A* and *B*); the observed restoration to the preintoxication level of intact SNAP-25 indicated loss of the protease activity. Similarly, type F-treated neurons regained the majority of their *Sbr2*, coincident with a fairly fast return to the initial level of exocytosis (Fig. 2, *C* and *D*). In both cases, exocytosis appeared to resume more rapidly from a partial blockade, with minimal inhibition of exocytosis and only traces of SNAP-25 or *Sbr2* cleavage being noted at 7 days (Fig. 2, *B* and *D*). Replenishment of intact SNAREs coincided with resumption of exocytosis. From the dose dependence, a half-life of the duration of inhibition ($t_{1/2}$) by each toxin was determined by monitoring reduction in the extent of blockade of exocytosis at different times after initial exposure to a given concentration of toxin. In the case of BoNT/E, the concentrations required to yield a 40% inhibition of exocytosis at 0, 2, and 4 days after the removal of toxin were 0.021, 0.22, and 0.80 nM, respectively (Fig. 2*A*). When these values were subjected to first order decay analysis, a $t_{1/2}$ of 0.70 ± 0.15 days (mean \pm S.D.; $n = 6$) was calculated; a second series of experiments yielded a comparable value. The mean of both experiments was 0.73 ± 0.11 days (mean \pm S.D.; $n = 12$; Table I). Similarly, analyses of data from two recovery experiments with BoNT/F yielded a mean $t_{1/2}$ of 1.76 ± 0.28 days (Table I). It is assumed that these $t_{1/2}$ values represent a combination of the times required for cellular removal of the toxin protease activity and synthesis of functional intact SNAREs.

BoNT/A-induced Blockade of Transmitter Release from Cerebellar Neurons Lasts Much Longer than That Caused by Type B—Neurons treated for 24 h with BoNT/A yielded dose-dependent inhibition of transmitter release up to a maximum of 65% blockade at 25 pM (Fig. 3*A*), accompanied by proteolysis of up to 90% of the SNAP-25 (Fig. 3*B*). More extensive inhibition of the Ca^{2+} -dependent release could not be achieved, even with 2 nM toxin (data not shown; see "Discussion"); it is notable that only 10 pM yielded ~50% blockade of the inhibitable component (Table I). After toxin was washed away, no significant recovery

TABLE I
Potencies and durations of inhibition of exocytosis by BoNTs in cerebellar granule neurons

| Purified toxigenic toxin | Concentration causing 50% blockade of transmitter release ^a | $t_{1/2}$ of inhibition ^b |
|--------------------------|--|--------------------------------------|
| BoNT/A | 10 | $>21^c$ |
| BoNT/B | 100 | 9.84 ± 2.12 |
| BoNT/C1 | 13 | $>25^c$ |
| BoNT/E | 43 | 0.73 ± 0.11 |
| BoNT/F | 1350 | 1.76 ± 0.28 |
| ToTx | 6.5 | Not studied |

^a These values were determined from detailed concentration dependency studies plotted in Figs. 2–4.

^b The $t_{1/2}$ is calculated by subjecting the time-dependent decreases of inhibition to first order decay analysis.

^c No significant diminution in the extent of inhibition was noted at the listed times, in several experiments.

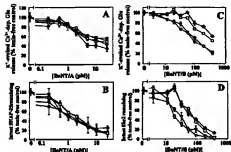


Fig. 3. Blockade of transmitter release by BoNTA remains undiminished for more than a month and correlates with persistence of SNAP-25_A inhibition by BoNTB partially recovered in this period coincident with incomplete *Sbr2* replenishment. Neurons (7–9 DIV) were incubated for 24 h in culture medium with the specified concentrations of BoNTA (A and B) or BoNTB (C and D). After removal of BoNTA by washing, neurons were tested immediately (0 days, 0), or the medium was replaced and the cells were cultured for 7 (V), 14 (VI), 21 (VII), or 31 days (VIII). In the case of BoNTB treatment, the neurons were maintained for 9 (V), 7 (VI), 25 (VII), or 26 (VIII) days after removing the toxin. A and C, the extents of blockade of evoked transmitter release were measured (as in Fig. 1 legend) and expressed as the means \pm S.D. ($n = 3$ or 4). B and D, equal amounts of neuronal protein were immunoblotted with anti-SNAP-25 C-terminal peptide lgs or anti-HV82 lgs (*Sbr2*), and the extents of SNARE cleavage were determined (means \pm S.D.; $n = 3$), as in Fig. 2.

from inhibition of release or replenishment of intact SNAP-25 was detected at any of the BoNTA concentrations employed (Fig. 3, A and B), upon weekly monitoring up to 31 days. Additional experiments also demonstrated a lack of significant recovery from blockade of exocytosis 1 month after toxin exposure (data not shown and detailed later). Therefore, the $t_{1/2}$ of BoNTA exceeds 31 days (Table I).

In contrast, BoNTB induced a concentration-dependent inhibition of transmitter release achieving ~80% blockade (Fig. 3C; 100 pM gave 50% inhibition; Table I), together with nearly complete cleavage of *Sbr2* (Fig. 3D), precluding *Sbr1* that is resistant (38). Notably, the truncated *Sbr2* fragments produced by BoNTB or BoNTF, potential contributors to the poisoning, were not visible on Western blots (see below). Upon removing BoNTB by washing, recovery from inhibition occurred in a time-dependent manner and was accompanied by equivalent partial replacement of intact *Sbr2* (Fig. 3, C and D). Exponential decay analysis of data from two BoNTB dose dependence recovery studies yielded a mean $t_{1/2}$ of 9.84 ± 2.12 days

(Table I). Therefore, compared with the long lasting BoNTA and short acting BoNT/E and F, type B exhibits an intermediate duration of inhibition.

BoNT/C1 Exerts a Long Lasting Inhibition of Exocytosis and Is Neurotoxic—Two days after a 24-h exposure to BoNT/C1, concentration-dependent cleavage of STX1 and SNAP-25 was observed (Fig. 4B). A concomitant inhibition of K⁺-evoked exocytosis occurred (Fig. 4A; 13 pM gave 50% inhibition; Table I) that correlates well with the proteolysis of SNAP-25 but not STX1 (Fig. 4B). Whereas there was extensive formation of a SNAP-25 product with a size corresponding to the known N-terminal fragment (residues 1–196) (Fig. 4D; see the Introduction), only a residual content of the toxin-truncated N-terminal STX1 product (residues 1–253) (reviewed in Ref. 3) could be detected (Fig. 4D). Short exposure to a higher concentration of BoNT/C1 (2 h) resulted in ~80% cleavage of STX1 and SNAP-25; however, the STX1_{1–253} fragment was no more abundant and only clearly visible if immunoblots were overdeveloped (Fig. 4D, asterisk); thus, this fragment is rapidly degraded. The possibility that this potential competitor of the SNARE complex contributes to the inhibition of exocytosis is therefore unlikely.

Closer examination of neuron abundance in control and BoNT/C1-treated cultures revealed a dose-dependent effect on survival with 0.33 nM yielding ~50% lethality within 2 days (Fig. 4C and Table I). Indeed, C1 lethality was much more apparent after 18 days exposure as indicated by the diminution of BoNT/C1-resistant markers, *Sbr2* and synaptotagmin (data not shown); also, direct neuron counting (Fig. 4C) revealed that ~60% of the neurons treated with 33 pM had died. It is apparent that neurons exposed to 10 pM BoNT/C1, showing 47.3 ± 5.3 and $7.7 \pm 9.6\%$ cleavage of SNAP-25 and STX1 at 2 days, survived well over the additional 18 days (Fig. 4, B and C), but those that experienced more extensive initial proteolysis of SNAREs fared poorly. For instance, only ~10% of neurons survived 18 days if their initial intact STX1 content had been diminished by $48.0 \pm 8.0\%$ (Fig. 4, B and C). Despite the difficulties experienced with neuron survival, it was still possible to demonstrate that neither significant recovery from the dose-dependent inhibition of exocytosis (Fig. 4A) nor increased contents of intact SNAP-25 and STX1 occurred (Fig. 4B). Additional separate experiments lasting either 18 or 25 days post-toxinization (Table I) also demonstrated a lack of significant recovery from BoNT/C1-induced blockade of neuroexocytosis.

[³⁵S]Methionine Pulse Labeling Demonstrates That BoNT/A Protease Has a Long Lifetime in Central Neurons: SNAP-25_A Is Turned Over as Rapidly as the Intact Polypeptide—Failure to recover exocytosis from BoNTA-intoxicated neurons and persistence of SNAP-25_A suggested that the prolonged inhibition arose from an extended lifetime of SNAP-25_A (known to block exocytosis) (22, 23) and/or the continued activity of the toxin. To address the former possibility, the $t_{1/2}$ of SNAP-25_A in BoNTA A-pretreated neurons was assessed relative to the intact protein (Fig. 5). The cells were treated for 24 h with BoNTA and subjected to a 4-h pulse labeling before being harvested (i.e. 0 h chase) or chased for the specified times in label-free medium (Fig. 5). After immunoprecipitation of SNAP-25, fluorography revealed time-dependent decreases in [³⁵S]Met-SNAP-25 and -SNAP-25_A (Fig. 5, A and B). Additionally, immunoblotting of the precipitates with an anti-SNAP-25 antiserum indicated that equivalent amounts of SNAP-25 were analyzed (Fig. 5, C and D) and that the toxin had proteolyzed a substantial fraction in advance of pulse labeling (Fig. 5D). Less than 50% of the newly synthesized [³⁵S]Met-SNAP-25 was proteolyzed by BoNTA during the 4-h pulse labeling period (Fig. 5E; i.e. 0 h chase); this contrasts with the >85% cleavage of total SNAP-25

Fig. 4. BoNT/C1 potentially blocks transmitter release for many weeks because of a corresponding persistence of SNAP-25_{C1} and a reduced STx1 content; this serotype can cause cell death. Neurons (7–8 DIV) were incubated for 30 h in culture medium with the specified concentrations of BoNT/C1. After washing away the toxin, the medium was replaced, and the neurons were assayed 2 (filled symbols) or 18 (open symbols) days later. A, the extents of blockade of evoked transmitter release were measured (as in Fig. 1 legend) and expressed as the means \pm S.D. ($n = 3$ or 4). B, neuronal protein was immunoblotted using IgG specified in the Fig. 2 legend. The data (means \pm S.D.; $n = 3$) from densitometric scanning of blots were used to determine the extents of cleavage of SNAP-25 (2 days, \bullet ; 18 days, \circ) or STx1 (2 days, \blacktriangledown ; 18 days, \triangledown). C, neuron survival at 2 (filled) and 18 (open) days was assessed microscopically by counting viable cells. In D, following a 2–5 exposure in the absence or presence of BoNT/C1, equal amounts of protein were immunoblotted using the specified antibodies (an asterisk indicates the toxin-truncated product, STx1 1–253; this was only visible after a prolonged development time).

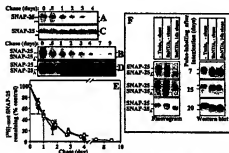
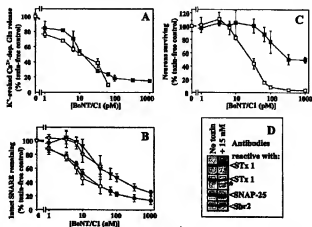


Fig. 5. [³⁵S]Methionine pulse labeling and immunoprecipitation determine the $t_{1/2}$ values of intact SNAP-25. In developing neurons and demonstrate that BoNT/A protease persists for at least 8 weeks. Neurons cultured for 7 DIV were exposed for 16 h in the absence (A and C) or presence (B and D) of 100 pM BoNT/A prior to [³⁵S]methionine pulse labeling (see "Experimental Procedures"). After the specified chase, the neurons were washed, detergent solubilized, and immunoprecipitated. B, SNAP-25 labeled SNAP-25 was subjected to SDS-PAGE and fluorography (A and B) and Western blotting (C and D) using an anti-SNAP-25 Ig of an alternate species to that used for immunoprecipitation to ensure equal contents in each sample. E, the SNAP-25 bands were excised, and their radioactive contents were measured by scintillation counting; the data (\pm S.D.) are plotted from three experiments each performed in duplicate or triplicate. \bullet , SNAP-25; \circ , SNAP-25A. F, neurons cultured for 7 DIV were exposed for 24 h in the absence or presence of 10 pM BoNT/A and then maintained in culture without toxin for the specified period, prior to pulse labeling (with or without chase) and immunoprecipitation of SNAP-25. Immunoprecipitated SNAP-25 was fractionated by SDS-PAGE, and the newly synthesized radiolabeled protein was analyzed by fluorography and Western blotting (see "Experimental Procedures").

detected immunologically (Fig. 5D); therefore, newly synthesized SNAP-25 can only represent a minor portion of total SNAP-25. Accurate measurement of radioactivity remaining in the SNAP-25 bands from multiple experiments by scintillation counting (Fig. 5E) revealed time-dependent decreases in SNAP-25₁ in BoNT/A-treated cells with decay kinetics comparable with the intact protein in toxin-free cells. Extending the chase period beyond 4–8 days revealed a diminution of almost

all of the residual [³⁵S]Met-SNAP-25₁ detected (Fig. 5, B and E); the $t_{1/2}$ values of SNAP-25 and SNAP-25₁ extrapolated were 0.89 ± 0.28 and 0.95 ± 0.20 days, respectively (Fig. 5E). Therefore, the $t_{1/2}$ of SNAP-25₁ does not account for the longevity of BoNT/A-induced inhibition (N.B. $t_{1/2}$ > 31 days), at least, in these cultured central neurons.

Next, the persistence of the BoNT/A protease was assessed by examining whether the newly synthesized SNAP-25 was still being proteolyzed at various periods after toxin exposure, visualized using pulse labeling and immunoprecipitation (Fig. 5F). Thus, neurons incubated for 24 h in the absence or presence of 10 pM BoNT/A (a concentration sufficient to yield a nearly maximal SNAP-25 cleavage) were cultured in the absence of toxin for the specified period prior to pulse labeling and isolation of SNAP-25, as outlined above (Fig. 5F). Additionally, toxin-treated neurons were chased for 14 h to allow sufficient time for the toxin to proteolyze new [³⁵S]Met-SNAP-25. Immunoblotting revealed $\sim 90\%$ cleavage of SNAP-25 in precipitates from type A-treated neurons at all periods examined (Fig. 5F). Importantly, 7, 15, and 20 days after intoxication, newly synthesized [³⁵S]Met-SNAP-25 was still efficiently proteolyzed, particularly following the additional 14-h chase (Fig. 5F). Reduced neuron survival precluded assessments longer than 3 weeks. Therefore, the notable longevity of BoNT/A-induced inhibition in these cultured central neurons results from persistence of its protease.

Co-exposure of BoNT/A-treated Neurons to Type E Failed to Shorten the Inhibition of Exocytosis: Removal of up to 28 Residues from SNAP-25 Did Not Alter Its Turnover—In view of the observed ability of BoNT/E to forego the paralysis time induced by type A at human and murine neuromuscular junctions (16), neurons were pre-exposed for 24 h in the absence or presence of 10 pM type A (Fig. 6A, hatched bars) or the latter plus 2 nM BoNT/E (Fig. 6A, cross-hatched bars) prior to assessment of blockade of transmitter release and SNAP-25 cleavage (Fig. 6B). The BoNT/A and E concentrations employed yielded nearly maximal inhibition (Fig. 6A) and cleavage of intact SNAP-25 (Fig. 6B); SNAP-25₁ predominated in doubly treated cells, consistent with the ability of type E to proteolyze SNAP-25₁ as efficiently as intact substrate (39). Following a 7-day recovery period, sufficient for nearly complete recovery from the 2 nM BoNT/E used (Fig. 2A), evoked release from the

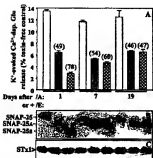


Fig. 6. Co-exposure of neurons to BoNT/A and BoNT/E does not accelerate recovery of exocytosis or deplete SNAP-25. *A*, neurons cultured for 8 DIV were incubated for 1 day in medium in the absence (open bars) and presence of 10 pM BoNT/A (hatched bars) or 10 pM type A and 2 nM BoNT/E (cross-hatched bars). After removal of toxin(s) by washing, the blockade of evoked release was measured at the specified times (means \pm S.D.; $n = 4$; see Fig. 1); the values in brackets represent percentages of inhibition of transmitter release relative to non-toxin-treated controls. The samples were blotted using anti-SNAP-25 (*B*, clone SMI-81) or anti-STX1 (*C*, clone HPC-1). STX1 immunoblotting confirmed that equivalent amounts of protein were used. The results are representative of two experiments.

BoNT/A- and E-treated neurons remained blocked to a similar extent as in cells exposed to BoNT/A only (~60% versus ~54% inhibition). Indeed, even 19 days after co-poisoning, the neurons retained the same level of blockade of release equivalent to that by type A alone (Fig. 6A; i.e., ~46 and ~47%, respectively). Consistent with the continued blockade of exocytosis by BoNT/A, SNAP-25 in the co-treated neurons existed predominantly in the A-truncated form (Fig. 6B); additionally, sequential application of BoNT/E up to 1 month after BoNT/A failed to alleviate blockade of exocytosis by the latter (data not shown).

Pulse-chase studies were performed to compare the turnover rate of intact and BoNT/E- or BoNT/C1-protected SNAP-25 in fully differentiated neurons (16 DIV) possessing maximal SNARE contents (Fig. 1C and data not shown), compared with immature neurons (Fig. 5). Fluorographs demonstrated that a 24-h pretreatment with either 4 nM BoNT/E or 10 pM BoNT/C1 yielded ~85 or ~50% proteolysis of SNAP-25 (Fig. 7A; because of the neurotoxicity of BoNT/C1 only submaximal cleavage was possible); immunoblots confirmed that equivalent amounts of SNAP-25 were present in each. Fluorography revealed that ~90% of the newly-synthesized SNAP-25 was rapidly proteolyzed by BoNT/E during the 4-h pulse (Fig. 7A; i.e., 0-h chase). Conversely, a 1-day chase was necessary for the low dose of BoNT/C1 to cleave the *de novo* synthesized SNAP-25. When the radioactive SNAP-25 remaining after the chase periods were expressed relative to 0 day chases for intact SNAP-25, SNAP-25_Δ, or SNAP-25_{CT}, all exhibited similar decay rates ($t_{1/2}$ of ~2 days) that were notably longer than that of SNAP-25 in younger neurons (Fig. 5).

The Rates of Replacement of Truncated Sbr2 and STX1 in Cerebellar Neurons Are Not Primarily Responsible for the Intermediate or Long Inhibition Exhibited by BoNT/B or BoNT/C1—Because prolonged inhibition by BoNT/B or BoNT/C1 may have arisen from slow rates of replacement of cleaved Sbr2 or STX1, this possibility was examined. Because mature neurons exhibit maximal SNARE contents from ~13 DIV onwards (only diminished by gradual loss of cell numbers; Fig. 1C and data not shown), the rates of SNARE synthesis and degradation must be equivalent in mature neurons (see "Discussion"). Therefore,

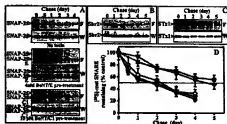


Fig. 7. Determinations of the $t_{1/2}$ of intact, and BoNT/E- or BoNT/C1-truncated SNAP-25, Sbr2, and STX1 in fully differentiated neurons. Cerebellar granule cells cultured for 16 DIV were exposed for 16 h in the absence or presence of the specified BoNT prior to [³⁵S]methionine pulse labeling (see "Experimental Procedures"). After the specified chase, the neurons were washed and detergent-solubilized, and their ³⁵S-labeled SNAREs were isolated by immunoprecipitation, using either SMI-81 IgG (*A*), SNAP-25_Δ anti-HV69 IgG (*B*; Sbr2), or HPC-1 IgG (*C*; STX1). The samples were subjected to SDS-PAGE and fluorography (*F*) or Western blotting (*W*) to ensure equal SNARE contents in each sample, using anti-SNARE antibodies generated in species different to those used for immunoprecipitation. Following fluorography, the SNARE bands were excised, and their radioactive contents were measured by scintillation counting; the values for SNAP-25 (*B*), SNAP-25_Δ (*C*), SNAP-25_{CT} (*W*), Sbr2 (*V*), or STX1 (*W*) are expressed (*D*) relative to their appropriate chase-free controls (means \pm S.D.; $n = 8$ or 4).

measurement of the $t_{1/2}$ of Sbr2 and STX1 would also indirectly indicate the rate of SNARE synthesis. Multiple assessments of equivalent immunoprecipitated SNARE samples from different chase periods revealed time-dependent decreases in radiolabeled Sbr2 and STX1, using fluorography (Fig. 7, *B* and *C*) and scintillation counting (Fig. 7D). A $t_{1/2}$ of 4–8 days was recorded for Sbr2; because the longest chases employed (5 days) failed to yield a 50% reduction of radiolabeled STX1, the $t_{1/2}$ can only be estimated as ~6 days.

The N-terminal Products from BoNT/B and BoNT/F Cleavage of Sbr2 Are Short-lived: the Stability of BoNT/F Protease Underlies Its Intermediate Duration Blockade of Transmitter Release—Residues 1–76 and 1–58 from Sbr, produced by cleavage with BoNT/B or BoNT/F, can bind tightly to STX1/SNAP-25 heterodimers *in vitro* (12, 13). It is therefore possible that they could prevent binding of intact Sbr2, result in competitive inhibition of ternary SNARE complex, and thus block transmitter release. For that reason, it was relevant to examine whether these products can evade cellular degradation and persist. Previous immunoblot analyses of Sbr in toxin-treated synaptosomes, cultured neurons, and neuroendocrine cells failed to detect the cleaved products (Figs. 2 and 3) (26, 38, 40), presumably because of rapid disposal or lack of detection. To evaluate these possibilities, intact recombinant Sbr2 was purified and incubated with BoNT/B *in vitro* before being subjected to SDS-PAGE and visualized by protein staining or Western blotting (Fig. 8A). As expected, BoNT/B produced two fragments (Fig. 8A). Signals were obtained for intact and Sbr2_{1–76} (Fig. 8A) using a polyclonal anti-Sbr antibody most reactive toward residues 33–45 (40). Sbr2_{1–136} was not retained on the nitrocellulose (Fig. 8A) and therefore could not be studied. Treatment of neurons with BoNT/B or BoNT/F was found to proteolyze Sbr2 as reflected by decreased Sbr immunoreactivity, but no lower *M_r* bands were detected, suggesting that these fragments are short-lived; thus, their contribution to the exocytotic blockade is precluded.

Having excluded the persistence of potentially inhibitory Sbr2 products, as well as slow Sbr2 replacement, being the reasons for the prolonged duration of BoNT/B, the longevity of

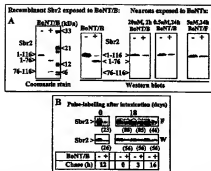


Fig. 8. The N-terminal products of Ssr2 proteolysis by BoNT/B or BoNT/E are short-lived in neurons whereas the BoNT/B protease persists for many weeks. **A**, recombinant purified Ssr2 was incubated with or without BoNT/B using conditions detailed elsewhere (40), fractionated by SDS-PAGE, and visualized by protein staining (2 μ g) or transferred to a nitrocellulose membrane (0.1 μ g) for Western blotting, using anti-Ssr2 Ig (anti-HY6; see "Experimental Procedures"). Primary Ig binding was visualized as in Fig. 1. Additionally, the neurons were treated in the absence or presence of BoNT/B or/and (specified), and equal amounts of protein (20 μ g) were Western blotted as outlined above. **B**, neurons cultured for 7 DIV were exposed for 24 h in the absence or presence of 100 pM BoNT/B and then maintained in culture without toxin for the specified period prior to pulse labeling (with or without the specified chase) and immunoprecipitation of Ssr2 (as in Fig. 7). Immunoprecipitated Ssr2-containing the newly synthesized radiolabeled component was analyzed by fluorography (**F**) and Western blotting (**W**). The bracketed values indicate the amounts of the intact Ssr2 immunoreactivity or radioactivity remaining in BoNT/B-treated samples relative to controls.

its protease was directly examined using pulse labeling and immunoprecipitation. Neurons exposed for 24 h in the absence or presence of 100 pM BoNT/B were pulse labeled immediately (0 days) or cultured for 18 days prior to pulse labeling and Ssr2 isolation. This treatment caused $76.2 \pm 3.9\%$ (mean \pm S.D.; $n = 4$) and $76.2 \pm 5.0\%$ (mean \pm S.D.; $n = 4$) proteolysis of Ssr2, immunolabeled or radiolabeled; the values for intact Ssr2 remaining from a typical experiment are shown in Fig. 8B. After 18 days, the amounts of Ssr2 cleavage on Western blots noted for BoNT/B-treated neurons had diminished to $43.9 \pm 5.4\%$ cleavage (mean \pm S.D.; $n = 9$), consistent with its intermediate persistence noted earlier. Importantly, newly synthesized [35 S]Met-Ssr2 was still being proteolyzed in a time-dependent manner (Fig. 8B); minimal cleavage of [35 S]Met-Ssr2 occurred following 0- or 3-h chases ($11.6 \pm 3.8\%$ and $14.1 \pm 8.7\%$, respectively), but after a 18-h chase, significant ($53.8 \pm 11.5\%$) cleavage of Ssr2 was evident (Fig. 8B; values are the means \pm S.D.; $n = 4$). Therefore, persistence of the toxin protease activity is the primary determinant of the longevity of BoNT/B-induced inhibition of exocytosis.

DISCUSSION

The detailed pulse-chase study of native and BoNT-cleaved SNAPs reported herein provides the first unambiguous and direct demonstration of a persistence of BoNT/A protease in central neurons, together with convincing evidence that it is the major factor responsible for prolonged inhibition of neuroexocytosis. Unexpectedly, SNAP-25, exhibited the same turnover rate as the full-sized protein in cerebellar neurons, in contrast with its reported persistence (2, 24)² in peripheral motor nerve endings. Apparently, an exceptional situation must exist in motor nerve terminals *in vivo* (discussed in Refs.

2 and 22),² allowing SNAP-25 to squat at the presynaptic membrane because co-treatment of human or murine endplates with BoNT/A and BoNT/E causes a rapid recovery, equivalent to that of BoNT/E alone (18).² The latter would seem to exclude an adequate level of toxin protease persisting—but another study did not detect such a rescue although different conditions (e.g. higher toxin dose) were used (41)—though perturbation of the otherwise persistent BoNT/A protease activity or localization following treatment with BoNT/E cannot be precluded. Notably, BoNT/A protease persisted unabated for longer than 1 month in cerebellar neurons, thereby precluding BoNT/E-mediated rescue of exocytosis or depletion of SNAP-25; the apparent lack of replacement of the latter has been observed previously for spinal cord neurons in culture, although SNAP-25 turnover or protease longevity were not directly measured (25). Similarly, a study performed on cultured neuroendocrine cells observed negligible recovery of catecholamine release or replacement of SNAP-25 over 2 months following BoNT/A treatment, apparently resulting from protease persistence (22). Therefore, SNAP-25, but not the E-truncated protein, is retained in motor nerve terminals *in vivo* at the synaptic vesicle release sites; this intriguing dissimilarity with peripheral and central neurons *in vitro* warrants further investigation.

Despite the obvious differences that exist between central cerebellar neurons and motor nerves, many similar neuronal characteristics are conserved; these include common exocytotic mechanisms and proteins, neurite extension, and synapse development. Also, our data reveal that picomolar concentrations of several BoNT serotypes block exocytosis when directly applied to central neurons in culture with potencies matching that observed for motor nerve terminals. *In vivo*, this has not been observed because toxin access to central and nonmotor spinal neurons is largely prevented by anatomical barriers (e.g. the blood brain barrier). Moreover, BoNTs do not exhibit detectable levels of retrograde transport, characteristic of TeTx. Preliminary unpublished studies comparing BoNT potency in cultured central neurons and motoneurons have indicated that BoNTs poison cholinergic nerves more rapidly. However, if toxin exposures are performed overnight (i.e. when the rate of toxin internalization is not the limiting factor), comparable potencies were observed in both cell types. Most importantly, however, for the purpose of this study concerned with the bases for the different longevity of BoNT serotypes, their relative lifetimes in these neurons are remarkably similar to the distinct durations of neuromuscular paralysis observed *in vivo* for rodents (see the Introduction).

Generation of an avid antibody specific for the LC protease of BoNT/E has allowed tracking of the minute quantities that remain after exposure to nanomolar concentrations. Immunoblotting of cell extracts, after a 2-h treatment with BoNT/E, for several chase periods up to 3 days later revealed that the majority of BoNT/E LC remained as a covalently linked di-chain, inconsistent with its delivery to the cytosol (where it would have been reduced). Therefore, there are at least two pools of toxin in these neurons: endosomal and cytosolic. Although it was necessary to use concentrations of toxins supermaximal to those needed to inhibit exocytosis, nevertheless, the $t_{1/2}$ values shown herein correspond to a $t_{1/2}$ of ~16 h obtained for cell-associated BoNT/E LC immunoreactivity (data not shown).

The different degradation rates found herein for SNAP-25 in developing and mature cerebellar granule neurons (~1 and 2 days, respectively) accord with data from earlier studies (42), which showed that the accumulation of SNAP-25 during development of neurons results from both increased expression and

reduced rates of degradation, processes that stabilize by 14 DIV. The $t_{1/2}$ values of Sbr2 and STx1 in mature neurons (~4.5 and ~6 days) are reported for the first time. These collective findings allowed consideration of the contribution that truncated SNARE replacement makes to the different durations of transmitter release inhibition by BoNT serotypes. Indeed, the results suggest that the rate of SNAP-25 synthesis governs the length of BoNT/E-induced inhibition. Interestingly, removal of up to 26 C-terminal residues from SNAP-25 does not alter its degradation rate, implicating other signals for regulation of its turnover. The rates of synthesis and degradation of Sbr2 must be more rapid in developing neurons relative to the much longer $t_{1/2}$ of 4–5 days observed for the fully mature protein (i.e., analogous to SNAP-25), because a $t_{1/2}$ of ~2 days was found for BoNT/F in developing neurons. Because another Sbr-cleaving toxin, BoNT/B, persists for much longer ($t_{1/2}$ ~10 days) than the periods required for SNARE synthesis or degradation of the truncated N-terminal fragment, persistence of its protease must account for the prolonged inhibition of exocytosis.

Recent work (43) highlighted the potential risks associated with the clinical use of large quantities of BoNT/B for achieving paralysis of medium length, because of a much reduced safety margin relative to BoNT/A. Although the $t_{1/2}$ values determined herein are dependent upon both the times required for removal of the BoNT protease and replacement of cleaved SNARE with intact, protease persistence primarily dictates the larger $t_{1/2}$ values measured in neurons treated with BoNT/A, BoNT/C1, or BoNT/D. Attempts by others to examine the $t_{1/2}$ of the LC of the closely related Clostridial neurotoxins, TeTx, in cultured spinal neurons, found that a highly radio labeled toxin disappeared long before even an initial onset of recovery from blockade of neurotransmission (44); the authors correctly suggest that degradation of TeTx LC ($t_{1/2}$ ~6 days) may underlie the slow recovery from neuroinhibition. Indeed, it has been estimated that only 10–100 intracellular toxin molecules are required to inhibit exocytosis (45), precluding straightforward radiolabeled detection; furthermore, this approach does not distinguish between relevant functional toxin protease in the cytosol and that which may reside in other cellular locations (i.e., endosomes). Therefore, the methodology used herein for measuring the kinetics of recovery from inhibition offers obvious advantages.

Detailed BoNT dose dependence studies revealed good correlations between losses of intact SNAREs and inhibition of evoked transmitter release, providing a direct demonstration of their involvement in up to 90% of the Ca^{2+} -dependent evoked glutamate exocytosis measured. Note that microanatomical features of motor neurons *in vivo* are not reproduced by neurons in culture (including motoneurons), and they could play important roles in determining the duration, localization, and molecular basis of paralysis (2). However, an imperfect relationship was observed regarding SNAP-25_α content and inhibition of release (~30% of the total) is apparently mediated by SNAP-25_β, because it was reduced by sequential BoNT/E administration. A similar situation has been found in permeabilized neuroendocrine cells (39, 46) and synaptosomes (47).

A small number of patients are primary nonresponders to BoNT/A therapy; also, multiple administrations may gradually elicit immunity in a tiny minority of responders and limit the efficacy of treatment (reviewed in Ref. 14). Therefore, an alternative serotype with the potency and duration of type A is required. In this context, these studies have demonstrated that BoNT/C1 may possess such therapeutic potential (17), except that it has been reported to impair neurite/axonal growth and

cause cell death, an effect not ascribable to contamination (Ref. 20 and this work). From the present investigation, it seems that such BoNT/C1 toxicity may result from its proteolysis of STx1 because the dose dependence study revealed that only minimal cleavage of STx1 coincides with the lethal effects, whereas extensive SNAP-25 cleavage was not lethal; also, the SNAP-25_{1–256} fragment is known to be nonlethal (22). Additional proteolysis of one or more of the other five syntaxin isoforms reported (9) has not been excluded; only STx4 and STx5 are known to be resistant to BoNT/C1 (reviewed in Ref. 3). An essential nonsynaptic vesicle docking fusion role for STx1 in developing neurons is suggested by its notable abundance in immature cerebellar neurons, which are almost devoid of the other SNAREs and lack the functional Ca^{2+} -dependent exocytotic machinery (Fig. 1C). In conclusion, this first detailed examination of the molecular basis for the extended action of BoNT/A relative to shorter acting serotypes in neurons has provided novel information that should aid the extension of therapies as well as the development of countermeasures for botulism.

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Properties and Use of Botulinum Toxin and Other Microbial Neurotoxins in Medicine

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INTRODUCTION

The eminent physiologist Claude Bernard wrote in his classic work entitled *Experimental Science* (8), "Poisons can be employed as a means for the destruction of life or as agents for the treatment of the sick." He went on to explain how certain toxins and poisons were valuable tools for analyses of the most delicate phenomena of living structures. Although several toxic substances of plant and animal origin were used in medical practice during his time, in recent years a great multitude of poisonous substances from plants, animals, and microorganisms are now finding use in studies on animal physiology and some are used medicinally in humans.

In December 1989 the U.S. Food and Drug Administration licensed botulinum toxin type A as an orphan drug for the treatment of the human muscle disorders strabismus, hemifacial spasm, and blepharospasm in patients 12 years of age and older, by direct injection of the toxin into the hyperactive muscle. Botulinum toxin is also being used experimen-

tally for the treatment of a number of other dystonias and movement disorders (25, 98, 191). The use of the toxin for human treatment came about over 20 years ago through the collaborative work of Alan B. Scott and E. J. Schantz. The treatment of neurological disorders with botulinum toxin type A has opened a new field of investigation on the application of the toxin to nerve and muscle tissue in the human body.

Various microbial neurotoxins are being used to understand the physiology of the nervous system and may have potential value in the treatment of certain types of muscular disorders through modification of nervous stimulation of muscle activity. Well-characterized microbial neurotoxins for this purpose include the neurotoxic proteins from *Clostridium botulinum* and *Clostridium tetani* and the low-molecular-weight neurotoxins saxitoxin and tetrodotoxin, from certain species of dinoflagellates and bacteria (Table 1). These toxins affect muscular activity by their direct action on the nervous system; for example, botulinum and tetanus toxins affect activity by a presynaptic block of the release of neurotransmitters, and saxitoxin and tetrodotoxin do so by altering the action potential at the voltage-gated sodium channels of neurons. These toxins differ from many other

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TABLE 1. Approximate relative toxicities of microbial neurotoxins

| Toxin | Minimum lethal dose in mice ($\mu\text{g/kg}$) | Moi wt |
|--------------------------------------|--|---------|
| Botulinum toxin, type A, crystalline | 0.00003 | 900,000 |
| Tetanus toxin, crystalline | 0.0001 | 150,000 |
| Saxitoxin (free base) | 9 | 299 |
| Tetrodotoxins | 8-20 | 319 |

microbial toxins such as diphtheria and cholera enterotoxins in that they exhibit relatively little cytolytic or cytotoxic activity. This review describes properties of presently known neurotoxins that are obtained from microorganisms and that, through their physiological action, may be valuable in medicine and pharmacology, particularly botulinum toxin type A, the only toxin which is presently being used for the treatment and relief of several human dystonias. It also points out the need for research on methods for culturing, purification, genetic expression, and preservation of these toxins applicable to their use for human treatment.

MICROBIAL NEUROTOXINS THAT BLOCK NEUROTRANSMITTER RELEASE

Properties of Botulinum Toxin Type A Relevant to Its Use in Medicine

Developments leading to the use of the toxin for human treatment. Botulinum neurotoxins are produced by certain strains of the bacterial species *C. botulinum*, *Clostridium butyricum*, *Clostridium baratii*, and *Clostridium argentinense* (86). The toxins are classified into seven serotypes, A through G, on the basis of their immunological properties. The botulinum neurotoxins comprise a family of pharmacologically similar toxins that block acetylcholine release from peripheral nerves and cause a flaccid paralysis. All of the serotypes of toxin can poison humans and other animals, but type A has caused the severest illness and many deaths from food-borne botulism and is the best-characterized botulinum toxin. Crystalline type A toxin is the serotype that is currently being used in therapeutic applications. The following sections describe the basic properties of botulinum toxin type A and the development of the toxin as a drug.

Investigations into the use of botulinum toxin type A for the treatment of hyperactive muscle disorders originated over 20 years ago through a fortunate set of circumstances and the ingenuity of Alan B. Scott, a surgeon at the Smith-Kettlewell Eye Research Institute in San Francisco. He contacted one of us (E.J.S.) regarding the availability of a toxic substance that might be injected into a hyperactive muscle and thus serve as an alternative to surgery for the treatment of strabismus, a condition in which the eyes are out of alignment. In my research on microbial toxins I had on hand highly purified crystalline type A botulinum toxin, produced by *C. botulinum*, and saxitoxin, the potent poison produced by the dinoflagellate *Gonyaulax catenella*. The mechanisms of action of these toxins had been known for many years (29, 33, 63, 103), and their possible use in the treatment of a hyperactive muscle was apparent but had never been tested. No record of such use in animals or humans was available. Both botulinum toxin and saxitoxin cause flaccid paralysis of skeletal muscle as a result of action

on the nervous system. Botulinum toxin type A appeared to be the toxin of choice for human treatment on the basis of animal studies and accidental cases of human food poisoning in which the paralytic action on survivors lasted for many weeks whereas recovery from saxitoxin poisoning took only a few days for survivors. We therefore began our collaboration on this work by using botulinum toxin experimentally on rhesus monkeys, in which Dr. Scott surgically produced a condition similar to strabismus. With the properly determined dose of botulinum toxin injected into the more active muscle, proper alignment of the eyes was achieved.

After 10 or more years of successful experiments on monkeys, the FDA granted Dr. Scott permission to treat strabismus in human volunteers. Strabismus in humans is a disorder of vision due to turning of one or both eyes from the normal position for binocular vision and is caused by hyperactivity of one or more muscles controlling eye position. This condition in humans usually is corrected by surgery, which involves cutting away a sufficient portion of the hyperactive muscle to allow the eye to assume its normal position. Successful human treatment with the toxin involved injecting measured amounts of the toxin, under carefully controlled conditions using electromyography, directly into the hyperactive muscle pulling the eye out of alignment. Injection of botulinum toxin weakened the overactive muscle, enabling compensation by the weaker one and resulting in permanent eye alignment after a period of temporary paralysis (192). The clinical work was first reported by Scott in the 1980s (188, 189), and the properties of the toxin in relation to its use in medical treatment was reported by Schantz and Scott in 1981 (180).

Special considerations on the preparation and maintenance of botulinum toxin type A for human treatment. Although the original toxin on hand and that prepared for the monkeys was sufficient, the toxin to be used for the human trials had to be prepared under more specific conditions that would, from best judgment, meet approval by the FDA. Botulinum toxin is the first microbial protein to be used via injection for the treatment of human disease. There was no precedent for the use of a microbial toxin in this manner, and protocols for this work had to be implemented. The important considerations regarding the toxin were its purity and dose on injection. The production by culturing and the purification had to be carried out so that the toxin was not exposed to any substance that might contaminate the final product in trace amounts and cause undue reactions in the patient. These restrictions required culturing in simplified medium without the use of animal meat products and purification by procedures not involving synthetic solvents or resins. Another concern was the problem of long-term stability of the toxin so that a supply was always available. Dilution of the toxin from milligram quantities to a solution containing nanograms per milliliter presented a problem because of the rapid loss of specific toxicity on such great dilution. Toxin can be diluted in pyrogen-free water or saline if used immediately for treatment, but stabilization of the toxin for longer periods requires the presence of another protein such as gelatin or albumin (173, 177). Although the commercial botulinum type A product is prepared in the presence of human serum albumin, the use of human serum albumin presents potential problems in that certain stable viral agents carried through from donors could contaminate the toxin. These and other concerns about the preparation and use of the toxin for human treatment are reviewed and discussed in the following sections.

Mechanisms of action of botulinum toxin. The primary

structure of the neurotoxin is such that the resulting shape (secondary and tertiary structures) causes a highly specific binding and block of acetylcholine release at myoneural junctions. Botulinum toxin is toxic to all vertebrates through weakening of skeletal muscle, and death may come about through paralysis of the muscles of respiration. Van Ermenen (226) considered that the toxin acted on the central nervous system, but it was later shown that the action is peripheral rather than central (50, 60). Most early studies on the mechanism of action of botulinum toxin were carried out with type A crystalline toxin (29, 33, 79). Botulinum toxin blocks cholinergic transmission at all cholinergic synapses in the peripheral nervous system, but conduction along axons is not affected (79). The chemical denaturation lasts for several months, and recovery of neurotransmission and muscle activity requires sprouting of new nerve endings and functional connections at motor end plates. The biochemical mechanisms of botulinum toxin in skeletal neuromuscular preparations, brain synaptosomes, chromaffin cell cultures, spinal cord cell cultures, and *Torpedo* and *Aplysia* preparations have been reviewed within the last 10 years (45, 81, 137, 158, 168, 200).

Preparation and properties of botulinum toxin type A for clinical use. The Food Research Institute, University of Wisconsin, has been involved in the production of crystalline toxin pertaining to food safety for many years, and small amounts of this toxin were used for the work on monkeys. However, the toxin that was to be used for human treatment by injection required special considerations, and preparation and purity of the toxin became essential (180). The type A toxin Hail strain was chosen for production of toxin because it consistently produced high levels of toxin (1 to 4 million mouse 50% lethal doses (MLD₅₀) per ml of culture broth). It was originally obtained from J. H. Mueller of Harvard University (118), and was developed at Fort Detrick, Md., by screening for high toxin production. Toxin is produced in a nutritive medium consisting of a casein digest, yeast extract, and dextrose at pH 7.3. Following inoculation, growth is usually complete in 24 to 36 h, at which time cells undergo lysis. Complete lysis and clearing of the culture take 2 to 3 days. The toxin is liberated during lysis and is activated by proteases present in the culture broth that convert a poorly active protoxin to the highly potent toxin.

The first successful attempt at purification of type A toxin from culture broths was accomplished by Snipe and Sommer (203) at the Hooper Foundation at the University of California in 1928, when they showed that 90% of the crude toxin could be precipitated from the spent culture fluid by the addition of acid to pH 3.5. About 20 years later, Lammann et al. (113), starting with the precipitate, obtained the toxin in crystalline form, and then Duff et al. (59) improved the method; the improved method is the basis for the present procedure for purification. The purification of botulinum toxin type A in our laboratory for human use was designed to be carried out by the simplest procedures and avoided exposure to substances such as added enzymes or columns of synthetic resins, used in some methods, that could contaminate the preparation and be carried into the final injected preparations. It is briefly described as follows. The type A toxin in the spent broth was first precipitated by adjustment to pH 3.5 with acid; 90% of the toxin was recovered in the precipitate. The precipitate was washed with water, and the toxin was extracted with 1 M salt solution at pH 6.5 and reprecipitated with acid at pH 3.7. The toxin was extracted from this precipitate with 0.05 M sodium phosphate buffer at pH 6.8, precipitated in 15% ethanol at -5°C, redissolved in

phosphate buffer, and crystallized in 0.9 M ammonium sulfate. This simplified procedure yields, for example, 60 to 70 mg of small, white, needle-shaped crystals (0.1 to 0.2 mm in length) from a 12-liter culture (15 to 17% recovery). Recrystallization under the same conditions yields 20 to 25 mg of crystalline toxin.

The crystalline type A toxin contains 16.2% nitrogen and, as far as is known, is composed only of biologically active amino acids (32, 207) for both the neurotoxin and the nontoxic proteins. The isoelectric point of the crystalline type A toxin is pH 5.6. Under slightly acidic conditions, pH 3.5 to 6.8, the neurotoxic component of 150,000 *M_r* is bound noncovalently to the nontoxic proteins in such a manner as to preserve or help stabilize the second and tertiary structures upon which toxicity is dependent. Under slightly alkaline conditions (>pH 7.1) and in the blood and tissues of animals and humans, the neurotoxin is released from the toxin complex. RNA is also associated with the toxin complex but has no known role in activity or stability.

The molecular weight of crystalline toxin was initially shown to be 900,000 *M_r* on analysis in the ultracentrifuge at pH 3.8 to 4.4 (161, 173, 181, 229). Putnam et al. (161) showed that on electrophoresis, the crystalline toxin moved as a single substance with a molecular weight of 900,000. Lammann et al. (115) discovered that purified type A toxin could be separated into nontoxic and toxic components, when they found that a nontoxic component precipitated erythrocytes leaving the toxin in solution. Wagman and Bateman (229) also showed that the toxin moved in the ultracentrifuge as a single substance with a sedimentation coefficient of 19S at pH 5.6, but at pH 7.3 the toxin component (neurotoxin) dissociated and moved as a much smaller molecule (7S). Laiter DasGupta and Boroff (46) showed that at alkaline pH the neurotoxin could be separated from the nontoxic proteins by column chromatography.

On diffusion in agar gel at pH 4.2 the crystalline toxin moves as a single substance with a coefficient (*D*) of ca. 2×10^{-7} cm² s⁻¹ (161, 178). However, at pH 7.3, near to the pH at which the neurotoxin and nontoxic components dissociate, the diffusion rate of the neurotoxin increased to ca. 8×10^{-7} cm² s⁻¹, much higher than the rate expected for a globular protein molecule of 150,000 *M_r* (178). Diffusion depends to a great extent on the shape of the molecule, and the toxin may take on a threadlike structure that would diffuse faster than a globular structure.

The biological activity (toxicity) of the toxin, like many other biologically active proteins, is due to the spatial or conformational structure of the neurotoxin molecule (173, 182). The nontoxic proteins bound to the neurotoxin apparently play an important role in maintaining the toxic shape of the neurotoxin. Careful handling of purified toxin is therefore important for maintenance of stability. Botulinum toxin type A is readily denatured by heat at temperatures above 40°C, particularly at alkaline pH. Solutions of the toxin lose toxicity when bubbles form at the air/liquid interface causing stretching and pulling of the neurotoxin out of its toxic shape (173). This denaturation also takes place in an atmosphere of nitrogen or carbon dioxide. Dilution to extremely low concentrations (nanograms per milliliter) also tends to decrease the stability of the neurotoxin, but this can be prevented by diluting with a buffered solution (at pH 6.8 or below) containing another protein such as gelatin and certain albumins such as bovine or human serum albumin. When the pH is raised above 7.3, the neurotoxin is liberated, which is very labile. Because of its lability the neurotoxin is not practical for medical applications.

Crystalline botulinum toxin type A was the first microbial protein of this complexity to be considered an injectable substance by the FDA, and it was necessary to set down specifications for toxin quality. The following properties of the crystalline toxin obtained from many batches were found to be of the highest-quality toxin and were used for evaluation of batch 79-11, which was used in initial studies in humans and later licensed by the FDA: (i) a maximum absorbance at 278 nm when dissolved in 0.05 M sodium phosphate buffer at pH 6.8, (ii) an A_{280}/A_{278} ratio of 0.6 or less, (iii) a specific toxicity for mice of $3 \times 10^5 \pm 20\%$ MLD₅₀ per mg, and (iv) an extinction coefficient (absorbance) of 1.65 for 1 mg of toxin per ml in a 1-cm light path.

The purity of the crystalline toxin cannot be defined strictly in terms of percent purity because of small amounts of undefined material absorbing at 260 nm, most probably nucleic acid material, which associates with the toxin during culturing and is carried through the purification and crystallization procedures. This property is peculiar to certain crystalline proteins, in contrast to the crystallization of other simpler organic substances. We have based the quality of the toxin on obtaining as low an A_{280}/A_{278} ratio as possible, near to 0.55. Toxin from the first crystallization it should be reduced to about 0.55, which is considered representative of high-quality toxin. A third crystallization may reduce the ratio slightly but at a cost of yield, because only one-third to one-half of the toxin is recovered on each crystallization. Other crystalline proteins such as human and bovine serum albumins have absorbance ratios close to 0.5 (232). If it is assumed that the absorbing material at 260 nm is nucleic acid with an extinction of 20 per mg (12 times that of the toxin), the proportion of nucleic acid in a preparation with a ratio of 0.6 would be less than 0.1%.

Another test of purity and consistency for each batch of type A toxin is the banding pattern on solution electrophoresis and gel electrophoresis with crystalline toxin and reduced crystalline toxin. At or below the isoelectric point of 5.6, the toxin moves as a single homogeneous substance, of 900,000 M_r . Toxin reduced with sulfhydryl reagents shows the distribution after electrophoresis of the nontoxic components along with the neurotoxin subunits of 100,000 and 50,000 M_r . Electrophoresis carried out on several batches of crystalline toxin showed that the toxin is judged very similar for each batch (101).

The specific toxicity of a high-quality preparation of crystalline toxin should be 3×10^5 MLD₅₀ ($\pm 20\%$) per mg. The number of milligrams for this determination is based on the A_{278} , using the extinction of 1.65 to convert to milligrams of toxin ($A_{278}/1.65 = \text{milligrams of toxin } (\pm 3\%)$) (207). Because the immunological properties of type A toxin are independent of its toxic properties, the only means of evaluating the potency or acetylcholine-blocking power of the toxin is an animal assay (176). The mouse assay for toxicity determination may vary depending on the species of mice, their condition, and the conditions under which the assay is carried out. To minimize the variability, it is recommended that the mouse assay be carried out on any preparation used for human treatment with the use of a reference standard of type A toxin as described by Schantz and Kautter (177). There is no known chemical, physical, biological, or immunological test available that can replace the mouse test for toxicity evaluation.

An important factor in the medical use of botulinum toxin is a method of storage for retention of toxicity. The crystalline toxin formerly provided for reference in food assays was

dissolved and stored in 0.05 M sodium acetate buffer (pH 4.2) at 4°C (177), in which it retained toxicity for 1 to 2 years before a significant loss (20%) could be detected by mouse assay. The difficulty with storing the toxin in acetate buffer is that freezing causes complete detoxification and reliance on storage at 4°C without danger of freezing is not practical under certain circumstances. However, we have found that the most satisfactory method of storage is to leave the crystalline toxin at 4°C in the mother liquor of the second crystallization, in which toxicity was retained for 10 or more years. Retaining stability is important because it makes available a bulk supply of toxin to draw from over an extended period. When the FDA approved experimental trials on human volunteers, a large batch of crystalline toxin was prepared in November 1979 (designated 79-11) expressly for the human trials; 100 mg was supplied to Alan Scott and 50 mg was retained in storage at the Food Research Institute. This batch has been the sole source of botulinum toxin type A accepted by the FDA for human treatment and has been used by many physicians throughout the United States and some foreign countries. However, some loss in toxicity has occurred in batch 79-11, and we recommend that fresher batches of toxin periodically be prepared to avoid detrimental changes that may occur on aging. Crystalline type A toxin prepared in our laboratory does not appear to differ in potency or clinical efficacy from type A toxin prepared in England by using anion-exchange chromatography and RNase treatment (133, 222). However, we do not recommend the use of methods of purification involving enzymes, various exchangers, or synthetic solvents because of the chance of contamination.

Preparation of the toxin for dispensing as a drug and compatible for injection into muscle required (i) dilution in a suitable medium for stability of toxicity, (ii) filtration for sterility, and (iii) drying. Diluting a solution of botulinum toxin type A from a concentration of 1 or 2 mg/ml to nanogram concentrations causes detoxification unless another protein is added for protection. Gelatin at 2 to 3 mg/ml is generally used at pH 6.2 in the standard procedure for the mouse assay for toxin in foods (177). Bovine serum albumin has been used at 2 to 3 mg/ml in acetate buffer at pH 4.2 for good stability (177), and human serum albumin was adopted for medical use. Filtration in the presence of additional proteins can be carried out successfully to remove bacterial contamination without loss of toxicity. However, drying, which would have many advantages in long-term stability, under the conditions with human serum albumin at pH 7.3 resulted in a substantial loss (50 to 90%) of toxicity. This loss of toxicity is a very important consideration because of the possibility that the inactivated toxin will form a toxoid and immunize the patient against the toxin on continued use. Various methods of drying, particularly lyophilization, resulted in such losses. Experience with the toxin has proved that stability of toxicity is dependent on low pH (<7), but such low pHs are not compatible with injections into muscle tissue. A significant problem is the development of a medium and conditions to overcome the losses on drying, and research for this purpose is being carried out in our laboratory.

Therapeutic applications of botulinum toxin. Clinical studies have indicated that toxin injections can provide profound symptomatic relief for humans suffering from a wide variety of disorders characterized by involuntary movements of muscle groups (Table 2), particularly those involved in focal or segmental dystonias (25, 64, 92, 127). In 1911, Oppenheim (151) introduced the term "dystonia musculorum defor-

TABLE 2: Focal dystonias and involuntary movement disorders successfully treated with botulinum toxin type A*

| Condition | Symptoms of disease |
|------------------------------------|---|
| Strabismus | Crossed eyes |
| Blepharospasm | Spasmodic eye closure |
| Hemifacial spasm | Facial twitching and spasms |
| Eyeid disorders | Leeward turning of eyelid |
| Spasmodic torticollis | Abnormal movements or twisting of the neck and head |
| Oromandibular and lingual dystonia | Sustained mouth closure or lingual muscle contractions |
| Focal dystonias of the hand | Writer's cramp, musician's cramp, hand and arm muscle spasms |
| Spasmodic dysphonia | Uncontrolled vocal fold spasms |
| Other voice disorders | Vocal tremor, shattering |
| Neurogenic bladder | Abnormal urinary control; results from spinal cord injury |
| Anismus | Uncontrollable anal sphincter contraction |
| Limb spasticity | Occurs following stroke and other neurological disorders including cerebral palsy |

* Listed approximately in decreasing order of numbers of patients treated (25, 98, 179, 191).

mans" to describe children who had movement disorders such as twisted postures, bizarre walking with bending and twisting of the torso, and severe muscle spasms. Oppenheim pointed out that progression of symptoms often resulted in fixed postural deformities. Dystonia is currently defined as "a syndrome of sustained muscle contractions, frequently causing twisting and repetitive movements or abnormal postures" (64, 127). Dystonia can affect all regions of the body (127). Many patients with dystonias have been diagnosed as experiencing psychological stress and referred for psychological therapy (64), but were later found to suffer from specific neurological diseases (64, 127). Adult onset of focal or segmental dystonias (which affect only one or a few muscle groups) are more common than generalized dystonias (64, 127). A study in Minnesota estimated the prevalence of various dystonias to be 391 per million population (147). Focal dystonias may spread and lead to generalized dystonias, in which several muscle groups are involved. Focal dystonias progressed to generalized conditions in nearly 60% of affected children (onset before age 13) and in about 3% of adults (onset after age 20) (127).

Crystalline botulinum toxin has had great benefit in the treatment of involuntary muscle conditions, and injection of toxin is now considered the most effective treatment for a variety of focal dystonias (25, 98, 191). On injection the toxin acts directly or indirectly to alleviate conditions that result from muscle hyperactivity. Direct paralysis of target muscles is desired for certain indications including blepharospasm, torticollis, and other focal dystonias. Depending on the syndrome, toxin injection generally relieves undesired muscle movement for a few months, after which the abnormal movement returns and repeated injections are required. Paralysis of certain muscle groups can also lead to secondary desired effects (191). For instance, paralysis of a hyperactive muscle enables compensation by a weaker muscle, as in treatment of strabismus and certain limb muscle spasmodic disorders. In these conditions, the balancing of agonist and antagonistic muscle systems is the desired effect (191).

Strabismus was the first syndrome for which botulinum toxin therapy was introduced as an alternative to surgery

(188, 189, 192). Botulinum toxin is usually injected into the recti muscles with a Teflon-coated needle and electromyographic guidance to ensure accurate placement in the muscle; this is usually an office procedure. The toxin evokes a temporary denervation and muscle weakening, allowing the globe to return to normal alignment. Although botulinum toxin will not replace conventional surgical treatment, it has proved to be a useful adjunct to surgery in certain cases (189, 191).

Botulinum toxin is being used primarily for the correction of focal dystonias and other regional movement disorders. One syndrome approved for treatment is essential blepharospasm, in which persons suffer from involuntary eyelid closure. Blepharospasm is often accompanied by involuntary movements of head and neck muscles, a condition known as Meige syndrome (98). Meige syndrome manifests as uncontrolled blinking (blepharospasm) plus involuntary facial grimacing, frowning, facial contortions, spasmodic speech, and neck pulling (spasmodic torticollis) (24, 25). The age of onset of blepharospasm is often 50 to 70 years, and the syndrome may progress to other muscle regions. Injections of botulinum toxin type A into the orbicularis oculi muscle has given clinically significant benefit in 70 to 90% of more than 8,000 treatments (98). In most patients, the latency period from injection to onset of improvement was 2 to 5 days and relief persisted for an average of 3.5 months. The average dose was ca. 20 U (191). In some treatments, toxin diffused to neighboring muscles and caused temporary ptosis. Some patients have received repeated injections for 7 years or more, and no adverse long-term effects have been observed.

Hemifacial spasm is an often disfiguring syndrome characterized by involuntary movement of facial muscles controlled by the seventh facial nerve. Patients often find the movements disfiguring and socially and functionally incapacitating (25). Treatments with neuroleptic medications have been entirely ineffective. Injection of botulinum toxin (generally 10 to 20 U) has relieved hemifacial spasm in more than 90% of the patients treated. Most patients experience relief for 3 to 4 months, after which repeated injections have provided long-term relief in most individuals.

Spasmodic torticollis (cervical dystonia) is a dystonia affecting neck muscles and causing the head to involuntarily deviate in any direction (25, 75, 98, 208). It is among the most common dystonias, and the spasmodic contractions can cause posture deformity, head tremors, and pain. Over 1,000 cases of spasmodic torticollis have been treated, and the studies have reported improvement in 50 to 90% of the patients, depending on the dose and placement of the toxin. Comparatively large doses of botulinum toxin are used for injection at multiple sites. The larger quantities of toxin can diffuse to neighboring muscles, causing ptosis and other side effects.

Certain other diseases involving involuntary muscle movements have been successfully treated with botulinum toxin in a limited number of patients (reviewed in references 25, 98, 191, and 218). These include writer's and musician's cramps, hand tremors, spasmodic dysphonia and other laryngeal dystonias, neurogenic bladder as a result of spinal cord injury, spasms of the rectal sphincter (anismus), limb muscle spasms following stroke, leg spasms from multiple sclerosis, and spasticity in children with cerebral palsy. Botulinum toxin could potentially benefit humans who suffer from a variety of other hyperkinetic movement and muscle tone disorders including tics, tremors, bruxism, and pain brought on by muscle spasms (25, 98, 99, 218).

Although botulinum toxin is currently used for treatment of regional muscle groups, limited success has also been achieved with patients who suffer from hyperactivity of several muscle groups. Botulinum toxin has found limited use in tardive dyskinesia syndrome (221), a chorea marked by irregular dystonic movements and postures that can develop in mentally ill patients after treatment with neuroleptic medications. Some of these patients experience marked distress and suffer from disparate spasmodic disorders including repetitive blinking, backward arching of the head and trunk (retrocollis), rocking of the body, mouth grinding (bruxism), and involuntary voice sounds and grunting. In a pilot study, four patients were injected in diverse muscles and marked improvement was found in 2 weeks in all four individuals. Not all movement disorders in these patients improved, but several did including retrocollis, mouth control, and bruxism. Treatment of tardive dyskinesia syndrome by chemical denervation with botulinum toxin is complex because it involves different muscle groups. The strategy has been to focus toxin injection on the most involved muscle groups.

Generalized dystonias such as those observed in Parkinsonism present difficult problems for treatment because of the many muscles involved, but it is possible that if a proper method of administration could be worked out, these generalized conditions could be treated with toxin. One possible but untried route is the administration of low intravenous doses by which the toxin would spread regionally to many muscles.

Side effects of botulinum toxin. No adverse clinical effects of botulinum toxin have been found in patients who received low doses of botulinum toxin, e.g., ≤ 20 U. Single-fiber electromyography analysis has shown that injection of relatively large quantities of botulinum toxin (140 to 165 U) leads to toxin spread, weakening of distant muscles, and uncharacterized subclinical effects (116).

The primary side effect associated with local injections of botulinum toxin is weakening and ptosis of nearby muscles. One of the most prevalent and disturbing side effects is dysphagia, or the inability to swallow, and several patients have experienced upper airway obstruction after treatment with relatively high doses (>150 U) of botulinum toxin (25, 208). Dysphagia may be related to generalized weakness and inability to hold the head erect (75) or to weakening of muscles involved in swallowing. It may also be related to the dose and injection strategy used. To prevent dysphagia, Borodic et al. (23, 24) have recommended, on the basis of studies of toxin diffusion in tissues, the use of ≤ 100 U per treatment injected into several sites. Further research is needed to identify the lowest dose of toxin and sites of injections that will produce the desired control and prevent migration of toxin to neighboring muscle groups. Local side effects could be increased in patients who are being treated with drugs other than botulinum toxin that affect neuromuscular transmission (4).

There is interest among physicians in developing methods to prevent the spread of toxin to neighboring muscles. Scott (190) demonstrated that injection of antitoxin at the correct time following toxin injection partially prevented toxin migration. The currently available equine antitoxin could lead to undesirable reactions in some patients, and it would be valuable to have a source of human antibodies. In January 1991, human immunoglobulin G pooled from immunized human volunteers became available in a phase II clinical trial by the Orphan Drug Program of the FDA as a potential treatment for infant botulism (70). A similar pool of human

antibodies could also be useful to alleviate side effects of botulinum toxin injections without leading to patient reaction to the antiserum.

Changes in muscle tissue following botulinum toxin type A injections. Changes in skeletal muscles after botulinum toxin type A injection have been studied in animal models (53, 56, 57, 155). Duchene (56, 57) found that muscle fibers became atrophied and sprouting of nerve fibers was induced after injection of toxin into the leg muscle of mice. Sprouting of motor nerves was observed after 6 to 7 days and progressed for several weeks in the red soleus muscle; it occurred later in the predominantly white gastrocnemius muscle. Nerve sprouting occurred as complex branched arrangements which were apparently unable to establish functional connections for several weeks. The muscle fibers atrophied for 6 weeks or more and then increased in diameter to within normal limits within a few weeks. Changes in the localization and intensity of cholinesterase staining reflected the morphological changes. This work was important because it provided a new approach to quantitative characterization of reinnervation of denervated muscle. Pestronk and Drachman (155) evaluated motor nerve sprouting quantitatively after presynaptic blockade with botulinum toxin by measuring acetylcholine receptors with 125 I-labeled α -bungarotoxin. Muscle disease was maintained by repeated injections of tetrodotoxin. They showed that the amount of sprouting was correlated with the number of acetylcholine receptors and was greatest in the botulinum-poisoned muscles. Sprouting was inhibited by α -bungarotoxin, suggesting that the acetylcholine receptors had an important role in inducing sprouting and muscle reinnervation. These results suggest that the use of a combination of botulinum toxin and α -bungarotoxin could prolong muscle paralysis.

In an approach derived from that of Duchene (56, 57), Borodic et al. (23-26) have used the albino rabbit as an animal model to quantitatively determine toxin spread from the site of botulinum toxin injection. Acetylcholinesterase staining, muscle fiber size analysis, and ATPase staining were used to establish a denervation gradient. A gradient effect up to 30 mm from the site of injection of 2 to 3 U of botulinum toxin type A per kg was found with respect to morphological changes in muscle fiber size and histological staining. At distances greater than 30 mm, there was substantially decreased staining and much less muscle atrophy. Very similar results were found in a study with crude type B toxin (26). The denervation indicated by histochemical staining and fiber size analysis appeared transient and lasted for about 3 months for both type A and B toxins. By using muscle biopsies, innervation sites were also determined with humans (23-25). Borodic et al. (23) have also used electrical stimulation to determine motor points and optimal injection sites in botulinum toxin therapy.

Immunity to botulinum toxin. There is considerable concern about the possibility that patients will develop antibodies and become refractory to botulinum toxin treatment, particularly when relatively high levels of botulinum toxin are injected repeatedly over several years. The dose of toxin required to trigger antibody formation in humans is not known. The minute quantities of toxin ingested in food-borne botulism are not sufficient to evoke antibodies. Recurrent episodes of type B and type E botulism have been documented in the same individual, supporting the notion that repeated exposure to botulinum toxin may not impart long-term immunity (6, 186). Reported sensitivity to tetanus toxin in humans has also been reported (34).

Toxoid is commonly injected into laboratory workers to

stimulate antibodies and protect against accidents. The minimum dose of toxoid to elicit immunity in humans varies greatly with the individual and the toxoid preparation (3, 80, 197), but is probably similar to the immunological response to tetanus toxoid (73). Repeated injections of botulinum pentavalent toxoid after 0, 2, and 12 weeks and yearly boosters gave final titers of 3.2 IU of anti-A antibodies, 0.4 IU of anti-B antibodies, and 2.5 IU of anti-E antibodies per ml in a man (80). Antibodies were slow to develop, and a steep rise in the level of anti-A antibodies occurred in the fourth year of immunization. In an investigation of 77 patients subjected to the current U.S. schedule of toxoid injection at 0, 2, and 12 weeks, Siegel (197) reported that neutralizing antibodies to type A and B toxins were low or absent after the 12-week shot and significant titers were present only after yearly boosters. After the first booster, 74 (96%) had an anti-A antibody titer of 0.25 IU/ml or more, and only 44 (57%) of the subjects had an anti-B antibody titer of 0.25 IU/ml or more. (1 IU is defined as the amount of antibody neutralizing 10,000 MLD₅₀.)

Antibody formation has been observed in a small number of patients injected with botulinum toxin (98, 191). To date, about 12 of more than 7,000 patients treated have developed antibodies to type A botulinum toxin. Six patients injected with 300 to 400 ng and one injected with repeated 100-ng doses within 30 days developed antibodies within 30 days (191). Antibodies have been demonstrated to reduce the beneficial effect of treatment (98). More work is needed to evaluate the incidence of antibody formation and other immunities in patients repeatedly treated with toxin over several years.

Properties and Uses of Serotypes of Botulinum Toxin Other than Type A

Seven known serotypes of botulinum toxin (A through G) have been isolated and characterized (213), and it is likely that types other than type A will be used clinically, particularly in patients who develop immunity to type A. Furthermore, evidence is accumulating to show that different types bind to different receptors and may have subtle differences in their mode of action and that they could therefore complement type A in clinical applications. In the following sections, we review various basic science aspects of the botulinum toxins, especially as they pertain to potential clinical applications.

Botulinum in humans. When botulinum toxin enters the circulation from contaminated food or infection, it can cause a severe paralytic disease. Types A, B, and E have most commonly been involved in human botulinum (168, 213, 215), and type F has been the causative type in at least two outbreaks of food poisoning (78). Symptoms and severity of botulinum differ depending on the serotype and amount of toxin ingested, suggesting possible differences in the mechanisms of intoxication (215). Clinical observations have indicated that type A food-borne botulinum is often more severe and associated with higher mortality than botulinum from other types (37, 52, 95). A rapid onset of neurologic signs indicates a more severe episode of the disease (38). Benign forms of botulinum in which the course of the illness is milder and longer lasting have also been reported, particularly for type B (43, 100, 109, 209).

Botulinum in humans generally manifests as a rapidly progressive symmetrical neuromuscular paralysis. Patients with botulinum generally stay mentally alert during the poisoning unless anoxia sets in (108). Sudden respiratory or

cardiac arrests and airway obstruction, leading to death, can occur (109). Cardiac effects of botulinum toxin in animals and in humans have been reported (114, 215).

Botulinum toxin most often initially affects eye muscles supplied by susceptible cranial nerves, and the first signs of botulinum are often blurred and double vision (215). As the paralysis progresses and peripheral nerves are affected, signs such as dry mouth, difficulty in swallowing, weakness in head and neck movements, and difficulty in breathing become apparent. In type A and B botulinum, loss of musculature control manifests as ptosis and drooping of eye muscles, hypoaffective gag reflex, and weakness in upper and lower extremities (95). Atypical symptoms including asymmetric or late-onset of neurologic signs, paresthesia, nystagmus, ataxia, and sensory abnormalities are not uncommon (31, 95, 215). Ingested botulinum toxin can paralyze all muscles of the body. Symptoms of botulinum sometimes last for months, and recovery requires reinnervation by new nerve terminal axons and end plates. Weakness and fatigability may persist for 1 to 2 years (215). Recovery in adults is generally complete (38), but there are reports of central nervous system involvement in infant botulinum (100a).

Since botulinum is rarely encountered, it can be difficult to diagnose rapidly. Electromyography is useful for detection of decreased amplitude of muscle action potential in weakened muscle (215), and since conduction along the nerve axons is not altered by botulinum toxin, the proximal motor nerve conduction rates and distal latencies are normal (42). Botulinum is confirmed by the demonstration of botulinum toxin in the patient's serum or stool or in suspect food by mouse assay and neutralization with type-specific antitoxin (52). Botulinum toxin has been found more often in the serum of patients with type E or B botulinum than with type A, possibly because of the greater affinity of type A toxin for tissue receptors.

The actual dose of botulinum toxin to cause food-borne intoxication in humans is debatable and depends on the individual, the source and type of toxin, and the amount ingested. Accidental cases of human botulinum from toxin-contaminated food showed symptoms of botulinum and occasionally death from as little as 0.1 to 1 µg (100 to 1,000 ng or 3,000 to 30,000 MLD₅₀) (134, 140, 183), but results were quite variable, probably because of individual variation in the amount absorbed and the stability of the toxin in the gut. More data on toxicity is available for lower animals and monkeys. The lethal dose of crystalline toxin type A in mice was 1.2 to 2.5 ng (0.03 to 0.07 U/kg) (76, 80) and was 0.5 to 0.6 ng/kg for guinea pigs and rabbits (76). Scott and Suzuki (193) determined that the intramuscular LD₅₀ for juvenile monkeys (*Macaca fascicularis*) was ca. 39 U/kg (ca. 1.25 ng/kg) of body weight. Herrero et al. (91) reported a similar lethal dose of 40 U/kg by intravenous injection in *Macaca rhesus*. In Gill's table of lethal amounts of bacterial toxins, he reported botulinum toxin to be the most potent toxin known for primates, the lethal quantity of type A toxin being 0.5 to 0.7 ng/kg of body weight for monkeys and ca. 1 ng (30 U)/kg for humans (76). Larger quantities of types C, D, and E may be required to cause death in monkeys, whereas less type B is required (76). No data on intravenous toxicity are available for humans for botulinum toxins, but humans are probably as sensitive as guinea pigs and would be expected to be about as sensitive as monkeys.

Toxin production by the various serotypes of *C. botulinum*. Use of the various types of botulinum toxin in medicine will require a plentiful source of the toxins. The production of type A toxin under controlled conditions by the Hall strain,

as used for the preparation of toxin for human treatment, gives a uniform crystalline toxin in high yields. The toxin complexes of the other types have also been obtained by culturing and purification and could be useful clinically. However, the strain, medium composition, and culture conditions affect the yields and structures of the botulinum toxins.

To obtain the greatest quantity and highest quality of toxin, it is essential to maintain strains of *C. botulinum* that consistently produce high levels of toxin. However, the bacterium has a frustrating tendency under laboratory conditions to gradually lose its ability to produce high levels of toxin. Lewis and Hill (118) reported that the Hall strain made decreasing quantities of toxin on successive subcultures. Huhtanen (96) also reported that strains of type A and B toxins frequently become nontoxicogenic during culture. A more complete understanding of the physiological and genetic factors that control toxin production will be valuable for the development of other types.

The highest levels of toxin in group I *C. botulinum* (proteolytic strains of types A, B, and F) are generally produced in cell populations that undergo rapid autolysis and do not sporulate (21, 27), although Siegel and Metzger (198) obtained titers of 6.3×10^7 U with the Hall strain in a fermenter without appreciable cell lysis. Toxin formation is poor during sporulation, and spores contain only small quantities of toxin (ca. 1% of that found in cytoplasm) (58, 77). Takumi et al. (216) reported the isolation of nontoxicogenic variants of *C. botulinum* type A that had enhanced sporulation. The strain used for production of type A, the Hall strain, sporulates very poorly. Therefore, encouraging vegetative growth and autolysis and discouraging spore formation may be important for obtaining good yields of toxin.

Toxin formation is controlled by nutrition in group I and II *C. botulinum* (119, 152). Arginine delayed autolysis, affected sporulation, and repressed toxin formation in group I *C. botulinum* (28, 154). Toxin formation was repressed about 10,000-fold in group I, including the Hall A and Okra B strains, when abundant arginine was available in the medium (152), probably owing to nitrogen repression of toxin gene expression. Protease was also decreased by arginine in group I *C. botulinum*. In group II *C. botulinum* (nonproteolytic strains of serotypes B, E, and F), tryptophan availability repressed toxin formation, probably also in response to nitrogen sufficiency (119). These results indicate that fermentation conditions and mutant strains could be developed for improved toxin production.

Significance of complexes on toxin quality. The strain and culturing conditions also affect the quality of toxin that is produced. Schantz and Spero (181) found that botulinum toxins of the different serotypes occur in spent cultures as large protein complexes. In the ultracentrifuge the sedimentation coefficients for the complexes were 19S for type A, 16S for type B, and 13S for types C, D, E, and F. Sugii and Sakaguchi (211) showed that high-molecular-weight toxin complexes occur naturally in foods. It is now known that each of the types of botulinum toxin produced in food or in culture are conjugated proteins ranging in molecular weight from 300,000 to 900,000, comprising a molecule made up of one or two neurotoxic units of about 150,000 *M*, noncovalently conjugated to nontoxic proteins (168, 181, 213).

The formation of toxin complexes is very important for use of the toxins in medicine because the nontoxic proteins play an important role in maintaining the stability of the neurotoxic units. Isolated neurotoxic units were poorly toxic

to mice when administered orally (169, 170, 210). Peroral toxicity increased with incremental association of the neurotoxins with the protective proteins (150, 168–170, 210). The larger (19S and 16S) complexes of botulinum toxin types A and B were more toxic by the oral route and more resistant to acid and pepsin than were the smaller complexes. The isolated neurotoxins were rapidly inactivated by these conditions. Variations in the toxicities of different strains also probably depend on differences in the structures of the complexes. Ohishi (150) found that the oral toxicities differed considerably for the toxins of certain type A and B strains of *C. botulinum*. Of five B strains, Okra B produced the most potent toxin by oral challenge in mice. The 16S complex of the toxin was 700 times more potent than the 16S molecule from strain NH-2. A hybrid composed of the neurotoxin from NH-2 and the nontoxic components from Okra increased the oral toxicity close to that of the native Okra toxin, probably by protection of the neurotoxin in the gastric and intestinal tracts.

The size of the complex formed in types A, B, E, and F depends on the medium for bacterial growth. It has been known for years that some foods such as vegetables have high botulinogenic properties (134, 212). Sugii and Sakaguchi (212) showed that type A and B *C. botulinum* produced the stable 19S and 16S high-molecular-weight complexes in vegetables, whereas they produced the less stable 12S complex in tuna and pork. Nonconjugated neurotoxin was not found in any of the food substrates. They found that addition of iron or manganese to the growth medium resulted in a higher concentration of small toxin complexes (12S and 16S) in type A *C. botulinum*, suggesting an influence of metals on the size and stability of the complexes.

Biochemical and genetic properties of the neurotoxin component. The biochemistry of purified botulinum neurotoxins, particularly type A toxin, has been studied in considerable detail, and authoritative reviews are available (45, 81, 213). Neurotoxins have been purified for all serotypes except for type G; the type G toxin has been purified to a protein complex of high toxicity, but further purification resulted in substantial loss of toxicity (126, 146). Within a given type of toxin and strain of producing bacterium, there may be considerable heterogeneity in molecular structure and antigenicity, giving a mosaic structure (139). The neurotoxins all have high specific toxicities, from 10^7 to 10^9 MLD₅₀/mg of protein (213).

All types of neurotoxins are synthesized as single-chain protein molecules of about 150,000 *M*, with low toxicity. The protoxins are released from the bacterium during culture (48). Those of proteolytic (group I) *C. botulinum* strains are cleaved by extracellular proteases into two-chain molecules consisting of an H (heavy) subunit of about 100,000 *M*, and a L (light) subunit of about 50,000 *M*, (45, 47, 48, 171, 196). Toxin preparations from nonproteolytic cultures require exogenous protease treatment for protoxin activation. The H and L chains are covalently linked by at least one disulfide and noncovalent bonds (45) and possibly a metal component (10, 11). The H and L chains of the neurotoxins can be separated by chromatography after treatment with dithiothreitol and urea (171). The isolated chains are not toxic by themselves but can be recombined under carefully controlled conditions to obtain active toxin (110, 123, 214, 230). Recently a chimeric toxin which retained considerable activity was prepared between the L chain of tetanus toxin and the H chain of botulinum toxin type A (230). Chimeric toxins composed of defined fragments, e.g., the H chain from botulinum toxin and the L chain from ricin, could be

valuable in medicine, but much work needs to be done on their formation and clinical testing.

During proteolytic cleavage the neurotoxins undergo a molecular change in shape that increases toxicity (48). The nicking region was recently reported to contain multiple target sites susceptible to more than one protease (45, 47). DasGupta and Dekleva have proposed that two peptide bonds in a short region are cleaved at different rates during maturation of type A toxin and that 10 amino acids are excised (45, 47). The control of proteolysis to increase stability could be useful in the preparation of the toxins for medicine, as has been achieved with tetanus toxin, and to obtain defined fragments for construction of toxins with desired properties.

The presence of metals in neurotoxins may affect their stability. Bhattacharyya and Sugiyama (10, 11) reported that chelators for iron and manganese inactivated purified type A botulinum toxin and tetanus toxin. Analysis of purified botulinum neurotoxin for metal content by neutron activation indicated that one atom of iron was present for each toxin molecule. It was suggested that metals may be involved in linkage of the H and L chains of botulinum and tetanus neurotoxins (10, 11). Kindler and Meager (107) found that metal availability in the culture medium affected the formation of toxin. Culturing *C. botulinum* in a medium containing EDTA did not inhibit growth but completely suppressed toxin formation. The biological activity of botulinum toxin may depend on a transition metal component, possibly Fe. The presence of metals could be important in maintenance of activity and protection from oxidation during drying and for long-term stability.

Recent genetic advances have increased our understanding of the structure and expression of the botulinum toxins. The genes coding for botulinum neurotoxins types A, B, and E are present in one copy on the chromosome in representative strains (14, 219). Genomic libraries of *C. botulinum* type A chromosomal DNA (strain 62A or NCTC 2916) were prepared on plasmids and transformed into *Escherichia coli*. For safety reasons, separate subfragments that were 2 kb or less in size and did not encode the entire neurotoxin gene were cloned. Open reading frames which encoded a sequence corresponding to a polypeptide of 1,296 amino acid residues, 149,425 M_r (14) or 149,502 M_r (219), were identified. The nucleotide sequences were in agreement with the partial nucleotide sequence reported by Betley et al. (9). The promoter of the BoNT/A gene was not transcribed in *E. coli*; this may have been caused by the frequent presence of codons in the promoter region that are not normally present in *E. coli*. Codon usage in the botulinum toxin gene was similar to that previously found for the tetanus toxin gene (61, 65, 66). Overall, 90.3% of the degenerate codons ended in A or U. An exception to the codon bias occurred for Lys codons, in which the frequency of AAA and AAG was nearly the same (24 AAA and 20 AAG) (219) compared with 98 AAA and 9 AAG for the tetanus toxin gene. In *C. botulinum*, AUG and UAA were translational initiation and termination codons, respectively, and strong bias was found for Arg and Ser codons. Binz et al. (14) found that the A+T content in the 5'-noncoding region of the type A and type E toxin genes was 80.4 and 80.3%, respectively, higher than in the coding regions, where 73.6 and 72.1% A+T were found. Examination of the upstream region indicated that transcription started 118 to 127 nucleotides upstream from the translation initiation site (14). Regions of dyad symmetry were demonstrated in the 3' noncoding region that may be involved in regulation of transcription. Binz et al. (14) con-

cluded that botulinum neurotoxin type A was translated from a monocistronic RNA and that the mRNA did not also encode the hemagglutinin and other nontoxic proteins of the natural toxin complex. Thompson et al. (219) also concluded that a single open reading frame was translated, giving only the neurotoxin protein.

The sequence of the type A neurotoxin gene indicated that botulinum neurotoxin A does not possess a signal peptide in the terminal coding regions, supporting the notion that it is not a secreted protein. Cys residues are conserved at positions 1060 and 1280 of botulinum (and tetanus) toxins, and Cys-454 occurs at the same position in *C. botulinum* type A, B, and E toxins and in tetanus toxin (61). Cys-454 is the sole Cys residue in the N-terminal region of the H chain and is probably involved in disulfide bridging of the L and H chains. Cys-430 is also located at an identical position in botulinum and tetanus toxin L chains. Sequence analysis of botulinum type A toxin indicated that the H chain of type A neurotoxin had six histidine residues arranged in a motif which the authors suggested could be involved in the biological action of the toxin, possibly penetration through the nerve membrane. The deduced amino acid sequences of botulinum toxins had about 33% homology to tetanus toxin, and the H chains showed higher homologies than the L chains (14, 219). No homologies were detected to other proteins including ADP-ribosylating clostridial toxins.

The DNA sequences have also recently been obtained for other botulinum toxins including type D, C₁, and E neurotoxins and the C₂ ADP-ribosyltransferase (13, 71, 72, 87, 105, 106, 157). Highly homologous regions were detected among the various neurotoxin gene sequences and tetanus toxin gene. The C₂ gene was found to be unrelated to C₁ and D neurotoxin genes.

Structure and properties of nontoxic proteins of the toxin complex. Relatively little is known concerning the biochemistry and genetics of the nontoxic proteins associated with neurotoxin in toxin complexes. The type A complex contains at least two nontoxic proteins, one of which has hemagglutinating properties (115). Strains of *C. botulinum* that do not produce hemagglutinin have been isolated, and these form smaller complexes (125 and 16S) than are normally found (195) (111, 135, 211). The *in vitro* addition of hemagglutinin to the 12S complex results in formation of a 19S complex with increased stability (111). Binding of the hemagglutinin was inhibited by a heat-stable, dialyzable substance that has not been isolated (211).

DasGupta (44) reported that the hemagglutinins of type A and B toxins were constructed through aggregation of two small units of about 15,000 and 20,000 M_r. Recently, Somers and DasGupta (204) studied nontoxic proteins from type A, B, C₁, and E toxin complexes. The proteins isolated from types A, B, and E had various degrees of hemagglutinating activity (Hn⁺), while the protein from type B had no hemagglutinating activity (Hn⁻). The type A Hn⁺ and type B Hn⁺ were serologically cross-reactive. Type A Hn⁺, type B Hn⁺, and type C Hn⁺ were isolated as large aggregates (220,000 to 900,000 M_r), which were separated into multiple subunits of $\approx 17,000$ M_r by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The type E Hn⁺ of 116,000 M_r did not aggregate. The sequences of the 10 to 33 amino-terminal regions of the 17,000, 21,500, 35,000, and 57,000 M_r subunits of type A Hn⁺ and type B Hn⁺ were determined. Each of the subunits had a unique sequence, indicating that the subunits were not homomers of smaller units. The subunits types A and B had remarkably similar

sequence identity; i.e., the 21,500 *M_r* subunits were identical and the 57,000 *M_r* subunits had 80% identity.

An understanding of the genetics of the hemagglutinin component of the toxin complexes is also developing. Oguma et al. (148) showed several years ago that the capacity to produce hemagglutinin in *C. botulinum* type C was transferred by phages either separately or together with toxin. Physical linkage of a hemagglutinin gene and toxin gene was confirmed, and it was shown that the toxin and hemagglutinin genes were transcribed in opposite directions. Trzaski et al. (224) cloned the gene encoding the main component of hemagglutinin produced by *C. botulinum* type C. The complete nucleotide sequence of the gene indicated that it encoded a protein of 33,000 *M_r*. At 62 bp downstream from the termination codon of the cloned 33,000 *M_r* subunit of type C Hn⁺ was an initiation codon followed by a coding sequence for at least 34 amino acids. Somers and DasGupta (204) found that the derived amino acid sequence of this open reading frame had 73 to 84% sequence identity with the 17,000 *M_r* subunits of type A Hn⁺ and type B Hn⁺ and significant similarity with the N terminus of type B Hn⁺. This observation raises the interesting possibility that genes for some of the subunits have a similar genetic arrangement and a common ancestral origin. It is interesting that a sequence homology has been proposed between tetanus toxin and the hemagglutinin of influenza virus (138), indicating a possible viral origin of the neurotoxin.

New findings in the mechanisms of action of the different types of botulinum toxin. Botulinum neurotoxins A to G are antigenically distinct yet have a number of structural and mechanistic similarities. All of the neurotoxin types cause a chemical denervation at the myoneural junction by inhibiting acetylcholine release. However, there appear to be subtle differences in the mechanisms of action of the neurotoxins. Toxin types A and E, type B, and type F apparently bind to distinct high-affinity receptor regions with similar affinities (K_D 10^{-9} to 10^{-10} nM) in synaptosomes and at murine neuromuscular junctions (20, 45, 62, 110, 128, 228, 233). Binding may occur in regions composed of sialosyl residues and protein (184) and may first involve low-affinity association of the H chain followed by high-affinity attachment. In addition to binding to different receptor regions on the nerve surface, botulinum toxin types A and B have been reported to affect neurotransmitter release differently (74, 137). Electrophysiological studies have shown that type A affects asynchronous neurotransmitter release, whereas type B does not (137). Furthermore, an increase in the intracellular Ca^{2+} concentration by ionophore treatment reverses inhibition by type A but not type B in synaptosome preparations (5), and aminopyridine more readily reverses type A than type B inhibition at the myoneural junction (74).

Both the H and L chains of the neurotoxin may be required for poisoning in invertebrate systems (123, 158). In mammalian peripheral motor nerve terminals, the L chain alone is active after it is internalized (15, 49). The precise mechanism of blockade by the L chain is unknown, but it must affect a general and important component of the secretory machinery in various classes of neurons. Botulinum toxin blocks the release of several classes of neurotransmitters at central and peripheral neurons (15, 120). Recently, it has been proposed that the L chain may act at an intracellular membranous or cytoskeletal site to inhibit neurotransmitter release (5, 120). Because of the extraordinary toxicity of botulinum toxin, it is likely that it has enzymatic activity and acts catalytically or triggers a cascade of events that decrease neurotransmitter release. The intracellular

substrate of botulinum toxin remains an elusive goal that is being pursued by several laboratories.

An objective in treatment of hyperactive muscles is to prevent possible systemic reactions which could result from spread of toxin through the blood. Antibodies could be used therapeutically by application to the injection site to help limit the diffusion of toxin and alleviate side effects such as ptosis (190), or it may also be possible to add the nontoxic H chain after toxin injection to block toxin binding to neighboring nerves. The most desirable approach to avoid spread would be to confine the paralyzing action within the presynaptic nerve. An interesting recent development is the finding that stabilized mRNA (3' polyadenylated and 5' capped) corresponding to the nucleotide sequence of tetanus toxin gene (L chain) injected into *Aplysia californica* cholinergic neurons in a bath depressed neurotransmitter release in less than 1 h (136). Similar results were found for mRNA of the L chain of botulinum A toxin, but only when the H chain was also added to the bath. The L chains of tetanus and botulinum neurotoxins were demonstrated to be synthesized in the presynaptic neurons, and onset of toxin action was slower than that of neurotoxins injected directly.

The subtle differences in botulinum toxin mechanisms among the various serotypes suggest that combinations of botulinum toxins could be more effective in clinical practice than any one type alone. Further work is needed to produce, stabilize, and test the clinical effectiveness of different types. Preliminary work indicates that types B (26) and F (187) are useful in controlling certain spasmodic muscle disorders.

Clinical use of pure neurotoxins compared with toxin complexes. Most recent information concerning the structure and pharmacology of botulinum toxin has been obtained with purified neurotoxins, but it is unlikely that these will be used in a clinical setting. The toxin complexes are much more stable than neurotoxins and can be diluted and formulated with retention of toxicity. Pure neurotoxins can be kept for several weeks to months in solution in the cold but are inactivated on dilution, formulation, and drying. No clinical trials on primates have been performed with purified neurotoxins.

Sellin et al. (195) reported that injection of 1 to 20 U of crystalline type A botulinum toxin into the lower hindlimb of the rat produced a paralysis that lasted for several days. In contrast, injection of more than 1,200 U of type B neurotoxin was required to produce paralysis. The duration of paralysis was compared after injection of 20 U of type A or 5,000 U of type B toxin. Type A toxin caused paralysis for up to 7 days after injection, whereas type B toxin caused paralysis for only 3 days and twitching became evident at 5 to 7 days. It was also reported (104, 194) that pure type A neurotoxin was much more effective than type B or F neurotoxin in eliciting lasting paralysis in the lower hindlimb of rats.

Tetanus Toxin

Tetanus toxin, like botulinum toxin, is produced by an anaerobic sporeforming rod that has a similar morphology to *C. botulinum* (86). Unlike botulinum toxin, tetanus toxin can enter into the central nervous system by retrograde intraxonal transport through motor nerves (17, 81, 132). It causes uncontrolled spasms of voluntary muscles by blocking the release of inhibitory transmitters including γ -aminobutyric acid and glycine (132). Tetanus toxin also has significant activity in decreasing acetylcholine release in cholinergic peripheral nerves when injected locally (54) and could possibly be used as an adjunct to or independently

from botulinum toxin for control of hyperactive muscles if the acquired immunity could be overridden. Tetanus toxin could also be used pharmacologically to transport substances to the central nervous system (17, 18). The biochemistry and pharmacological activities have been recently reviewed (7, 81). Here we consider aspects of the toxin that pertain to tetanus toxin production, stability, and potential use in medicine.

Crystalline structure. Tetanus toxin was originally purified by Pillemer et al. in the 1940s by precipitation methods. They obtained toxin crystals by carefully carrying out repeated precipitations in methanol and controlling the ionic concentration, pH, and toxin concentration (156). Tetanus toxin, like botulinum toxin, is a simple protein that does not contain lipid or carbohydrate (156, 165, 166). Unlike botulinum toxin, tetanus toxin does not occur complexed with protecting proteins and will not survive gastric passage or cause food poisoning in humans.

Although crystals of tetanus toxin were obtained in the 1940s by Pillemer et al. (156) from cold alcohol solutions, crystallization was not confirmed by others for several years. More recently, two-dimensional crystals of tetanus toxin have been isolated from ammonium sulfate solutions after incubation for several weeks at 4°C (40, 167). Robinson et al. (167) obtained two-dimensional arrays of native tetanus toxin formed at the interface between a solution of the toxin and a phospholipid monolayer containing ganglioside. Crystalline arrays were obtained only when all three components (toxin, phospholipid, and ganglioside) were present. The three-dimensional structure of tetanus toxin at 14-Å (1.4-nm) resolution appeared as an asymmetrical three-lobed structure that could interact with the phospholipid monolayer in two possible orientations (167). The analysis indicated that tetanus toxin is composed of differently shaped domains with different functions.

Biosynthesis and activation of tetanus toxin. Tetanus toxin is synthesized intracellularly as a single polypeptide of 150,000 M_r that is released from the cells on autolysis and is then modified by proteases present in the medium (81, 88, 89). The single-chain molecule is difficult to isolate (81), and proteolytic modifications of the toxin have caused considerable difficulties in the accurate characterization of the molecule. Single-chain toxin can be prepared from washed extracted bacterial cells (88, 162) and by inclusion of protease inhibitors and use of specific purification procedures (7, 165, 231). Purified preparations containing protease inhibitor can be stored for 4 to 6 weeks without proteolytic modifications and loss of toxicity (165).

Conversion of tetanus toxin to the nicked form increases toxicity (7, 81). *C. tetani* forms proteases that produce nicking in culture (231), but many other endoproteases will also activate the toxin (2). Three regions in the molecule are particularly susceptible to nicking (81, 129). Mild trypsin treatment of intracellular single-chain toxin yields two chains of about 95,000 and 50,000 M_r. The modified tetanus molecule is strongly held together by noncovalent bonds, and reduction of disulfide does not result in separation of the chains. Strong denaturants such as urea or SDS (81, 166) or purification techniques such as isoelectric focusing (2, 231) are required to dissociate the chains.

The H chain of tetanus toxin possesses a particularly susceptible region that can be cleaved with proteases such as trypsin or papain, yielding two fragments (B and C) (2, 89). The isolated H and L chains and fragments B and C are poorly toxic compared with intact tetanus toxin (2). Ahert-Hilger et al. (2) reported that the nicking sites contributing to

toxicity are located within a region spanning no more than 17 amino acids, and the N and C termini are not altered during the modification. The separated chains were reconstituted to active toxin. By reconstitution experiments, the L chain was demonstrated to possess the paralyzing activity in isolated nerve-muscle preparations. The H chain is required for toxin entry into the nerve tissues and for axonal transport (2).

Large-scale production of tetanus toxin. Tetanus toxin is produced in deep culture by methods similar to those described for botulinum toxin. J. Howard Mueller, Pauline Miller, and associates at Harvard Medical School developed the methods currently used for production of tetanus toxoid. They experienced much frustration in obtaining consistent quality and the high titers of toxin required for toxoid demand (121, 142-144). They realized the importance of medium formulation in obtaining good-quality tetanus toxin. "If it were only possible to grow the tetanus organism on a medium containing only chemically defined substances of low molecular weight, it should become a relatively straightforward matter to study and control the factors involved in toxin production, and to obtain a uniform product free from any possible antigenic material other than the specific substance desired." (141). An extensive study was carried out to identify factors controlling tetanus toxin formation (143). On fractionation of components of the medium, the basis of good production was determined to be present in a pancreatic digest of casein. The key to good toxin production by *C. tetani* was later determined to be limitation of histidine (144). Abundant free histidine drastically decreased toxin production, while its limitation strongly increased titers (144). Since histidine is required for growth of *C. tetani*, it was necessary to find a method to limit the nutrient without stopping growth. Mueller and Miller found that providing histidine-containing peptides (e.g., glycyl-histidine) or histidine esters (e.g., acetyl-histidine) stimulated toxin production. Latham et al. (117) developed a protein-free medium which is currently used for tetanus toxin production. Mueller and Miller also isolated a high-producing strain (the Harvard or Massachusetts strain) (142) that is still widely used by many laboratories.

Tetanus toxin synthesis was found to be repressed by the addition of excess amino acids to the medium (223). Meluhby (131) reported that glutamate addition to the Mueller and Miller growth medium decreased toxin formation but shortened the time necessary for autolysis. The results indicate that nitrogen nutrition controls toxin regulation in *C. tetani*. It is interesting that excess nitrogen also represses botulinum toxin synthesis in *C. botulinum* (119, 152). Careful adjustment of the levels of iron salts in the medium is also necessary for good tetanus toxin production (67, 142). The mechanisms of nutritional regulation and its importance in the biology of *C. tetani* and *C. botulinum* have not been further studied to our knowledge.

Tetanus toxin, like botulinum toxin, is produced in highest quantities by nonsporulating cultures (85, 145). Highly toxigenic cultures autolyzed thoroughly and did not form endospores during culture. During culture, tetanus toxin was present within the cell and was not released until cultures lysed (141, 162). As with *C. botulinum*, it appears that toxin formation is associated with autolytic growth and inversely associated with sporulation (145, 153). It would be of interest to determine whether specific transcription factors, e.g., sigma factors, regulate transcription of the toxin gene and whether these are preferentially expressed or activated in autolytic growth compared with sporulation.

Genetics of tetanus toxin. Tetanus toxin production has

been recognized as an unstable property for many years (68, 142). Attempts were made early to correlate the toxicity with the presence of bacteriophage. Phages were induced in *C. tetani* by treatment with mitomycin C, but induction did not affect toxin production (159, 160). Nontoxic mutants were readily isolated at high frequency (0.8 to 3.2%) from the Harvard strain A47 by treatment with various mutagenic agents including *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, UV light, and rifampin (85). Hara et al. (85) found that cured, nontoxic strains still carried phages and proposed that plasmids could be involved in toxigenesis. Laird et al. (112) showed that toxigenicity was associated with the presence of a single large plasmid in 21 strains of diverse origin. Nontoxic derivatives were isolated, and each strain lost its plasmid. Two naturally occurring nontoxic strains were examined, and one was free of plasmids while the other contained a single large plasmid (112). The strains derived from the Harvard strain all contain a plasmid of 49 kb, which had identical restriction nuclease digestion patterns (112). By construction of a pool of nucleotide probes corresponding to the *N*-terminal amino acid sequence of tetanus toxin, Finn et al. (69) located the tetanus toxin gene to plasmid-related sequences. Surprisingly, strains with deletions in the plasmid still hybridized to the probes, suggesting that toxin gene sequences were still present but were not expressed to active product.

Eisel et al. (61) used a pool of oligonucleotides (heptadecamers) made up of all possible DNA sequences for *N*-terminal amino acids 8 to 13 of fragment C of tetanus toxin and screened plasmid preparations from toxigenic and nontoxic variants of the Harvard strain. Overlapping sequences that spanned the entire toxin gene were obtained from eight clones. The DNA fragments encoding tetanus toxin specified an open reading frame of 1,315 amino acids of 150,700 *M_r*. The open reading frame begins with an initiation codon for methionine, but purified toxin, like botulinum toxin, possesses proline at its *N* terminus and contains serine at the *N* terminus of the H chain (61). The molecular weights of the H and L chains calculated from the amino acid sequence are 98,300 and 52,288, respectively. Partial sequences reported for peptide fragments from the L chain (166) are only partly consistent with those obtained by nucleotide sequencing. The discrepancy may be caused by the extensive proteolytic processing that tetanus toxin undergoes following cell autolysis.

Computer searches using the primary sequence of tetanus toxin have not revealed primary structural similarities with any proteins other than botulinum toxin. Analysis of the primary sequence has also provided evidence that tetanus did not evolve by duplication of sequences within the H and L chains, which was earlier suggested by the similarities in amino acid compositions of the H and L chains (217) and by immunological similarities probed with monoclonal antibodies (227).

The availability of cloned tetanus gene fragments has enabled the production of tetanus toxin fragments for potential use as vaccines. Makoff et al. (124) expressed tetanus toxin fragment C in *E. coli* as 3 to 4% of the total cell protein. However, the coding sequence for fragment C is A+T rich and contains several codons rarely used in *E. coli*. Production was improved by replacing the coding sequence by a sequence optimized for codon usage in *E. coli* (125). More efficient translation of the mRNA was the most important factor for the increased expression. When the modified coding sequence was combined with improved promoter strength, fragment C was expressed as 11 to 14% of the cell

protein. Halpern et al. (83) cloned the sequence encoding fragment C and showed that the fragment expressed in *E. coli* retained ganglioside- and neuronal cell-binding activity. Recombinant fragment C was purified in one step by affinity chromatography. Recombinant fragment C was also immunogenic in mice and elicited antibodies that protected against tetanus toxin challenge. The availability of recombinant fragment C should be useful for a variety of research applications and for production of toxoid.

Pharmacological and medical applications of tetanus toxin. Tetanus acts primarily in the central nervous system and causes hyperactivity of the motor system and a spastic paralysis. Under specific conditions, tetanus toxin also inhibits peripheral neuromuscular transmission, resulting in a flaccid paralysis (82, 130). Tetanus toxin resembles botulinum neurotoxin in its structure and mode of action (81). Botulinum and tetanus neurotoxins have significant homology at the amino-terminal regions of the L and H chains, suggesting that at least portions of the respective genes evolved from a common ancestral gene.

Similarities in structure of tetanus and botulinum toxins have also been demonstrated in serological studies. Antibodies to type C botulinum toxin cross-reacted with other botulinum toxin serotypes and also reacted with tetanus toxin (149, 225). Tsuzuki et al. (225) found that a monoclonal antibody raised against botulinum toxin type E cross-reacted with botulinum toxin types B, C₁, and D and with tetanus toxin. Halpern et al. (84) developed antibodies against defined regions of the tetanus toxin to identify regions shared by tetanus and botulinum toxins. Synthetic peptides that corresponded to different regions of tetanus toxin were prepared and coupled to bovine serum albumin, which were used to immunize mice. Eleven of 13 peptides elicited antibodies that reacted with tetanus toxin in an enzyme-linked immunosorbent assay. Of 10 anti-tetanus peptide antibodies that reacted well with tetanus toxin, 1 reacted with botulinum toxin types B, C₁, and E but did not recognize type A. This antibody was made with a peptide corresponding to the amino-terminal end of the tetanus L chain, suggesting that this region is important in intoxication and that its structure is conserved in the two toxins. The antigenic region may be shielded in the native toxin but exposed on denaturation. Halpern et al. (84) also tested human tetanus immune globulin and mouse anti-tetanus serum for cross-reactivity with botulinum toxin, but none was detected. These results suggested that native forms of tetanus and botulinum toxins have little common surface antigenicity. This conclusion was also reached by Tsuzuki et al. (225), who prepared 306 monoclonal antibodies against the L chain of botulinum toxin type E and found that only 1 reacted with the other botulinum toxin types and with tetanus toxin.

Tetanus toxin has the unique ability to enter into the central nervous system through motor neurons. Because of the ability to travel up motor nerves, tetanus toxin or nontoxic fragments could provide a unique neurotropic agent to transport substances to the central nervous system (16-19). A 45-kDa nontoxic fragment, B-II₀ (fragment C), that bound to toxin-binding sites on neuronal cell membranes and transported retrogradely from the axonal endings within the muscle to the motoneuronal perikarya was isolated (19). Bizzini et al. (18) constructed hybrid molecules consisting of the neurotropic fragment C and the I₀ fragment derived from tetanus toxin connected through disulfide linkage. The I₀ fragment was specifically carried to the central nervous system. Bizzini (17) also reported that fragment C

could compete with rabies virus for attachment to binding sites on neuronal cells and affected the rate of spread of rabies virus. Cloning and expression of high levels of fragment C should lead to further studies of targeted delivery to the central nervous system and possibly to control of virus infections.

Tetanus toxin also can act peripherally, causing a flaccid paralysis in the manner that characterizes botulinum toxin (54). H chains of both botulinum and tetanus toxins form channels in lipid bilayers (94). The H₂ fragment of the H chain of tetanus toxin was found to antagonize the action of botulinum toxin in phrenic nerve-hemidiaphragm preparations (201, 202). Tetanus toxin is about 2,000 times more toxic at central inhibitory nerves than at peripheral synapses (12, 54) and is about 1,000 times less toxic than botulinum toxin type A at the myoneural junction (82). Dreyer and Schmitt (55) proposed that tetanus toxin and botulinum toxin type A act at different sites in nerve inhibition of transmitter release. Botulinum toxins type B (74, 195), D (54), and F (104), but not A (74), appeared to act in a similar manner to tetanus toxin in affecting transmitter release from the myoneural junction. The combination of botulinum and tetanus toxins or the construction of chimeras could potentially be used to control neurological disorders.

MICROBIAL NEUROTOXINS THAT ALTER VOLTAGE-GATED SODIUM CHANNELS

Other microbial neurotoxins impair muscle activity in a way different from botulinum and tetanus toxins by their effect on the action potential at the sodium channel of a nerve axon. Saxitoxin and tetrodotoxin are two classical examples of microbial neurotoxins that block or close the passage of sodium ions through the channel. Toxins produced by other dinoflagellates also produce changes in the action potential at the sodium channel and are briefly described below.

Saxitoxin is a potent rapidly acting neurotoxin produced by the marine dinoflagellate *Gonyaulax catenella* (206) and some bacteria (102, 122). Like botulinum toxin, it was first observed as a food-borne toxin, causing food poisoning that occurred only at certain times from consumption of mussels, clams, and some other shellfish that are plankton feeders. Consumption of toxic shellfish results in symptoms described as numbness of the lips and fingertips within a few minutes followed by a progressive paralysis of the arms and legs along with the development of labored breathing and asphyxia. Death may occur within 2 to 24 h, depending upon the dose, from respiratory paralysis. After survival for 24 h the prognosis is good, and no lasting effects of the toxin have been observed. The oral dose that causes death from accidental consumption of toxic shellfish by humans is 1 to 4 mg (5,000 to 20,000 mouse units) depending upon the age and physical condition of the patient. A mouse unit (MU) is defined as the minimum amount needed to cause the death of an 18- to 22-g white mouse in 15 min, which is usually the maximum time in which death will occur (174, 205).

Saxitoxin was first purified and crystallized by Schantz et al. (172, 179), and its structure was determined by X-ray crystallography by Jon Clardy (175). Purified saxitoxin is a very hygroscopic water-soluble toxin and is described chemically as a tetrahydropurine base with pK_a s at 8.5 and 11.5. It has a molecular weight of 299 as the free base. It has no UV absorption above 210 nm. As the dihydrochloride salt it is a white solid that is stable in acidic solution but loses activity above pH 7. The paralyzing action of saxitoxin or its

binding at the receptor of the sodium channel depends upon the presence of a hydrated ketone group in a particular position in the molecule. Reduction of this group to the alcohol results in the loss of over 99% of the binding and paralyzing activity.

The neurotoxic action of saxitoxin is due to its specific binding, even at extremely low concentrations (10^{-9} M), at the sodium channel of excitable membranes and preventing the passage of sodium ions through the sodium channel, thus blocking an impulse. The action or binding is concentration dependent, and binding is reversible. Controlled application of the toxin has been suggested as a possible local anesthetic. The effective dose in animals is relatively close to the lethal dose, as indicated by the steepness of the response curve, and pharmaceutical companies have not pursued its use in humans. However, when saxitoxin is mixed in small amounts with many classes of anesthetics, the effectiveness of the anesthetic action is greatly extended (1). The addition of 1 μ g of saxitoxin to a 1% solution of a typical anesthetic (1 part in 10,000) such as procaine increased the time of effective action two- to threefold (1). The result is not an additive one (1). The addition of saxitoxin to procaine as well as to other anesthetic compounds will also reduce the dose required to obtain a desired effect. The reason for this unusual action of saxitoxin with anesthetic compounds has not been fully explained, but the molecule must play an important part in nervous function in the presence of other substances that act on the nervous system. Saxitoxin and tetrodotoxin have been important in the establishment and characterization of the sodium channel in myelinated and unmyelinated nerve membranes (90, 93, 102, 163, 164) and for the study of related diseases such as multiple sclerosis.

Another species of dinoflagellates, *Gonyaulax tamarens* var. *excrucians*, produces saxitoxin substituted with sulfite and sulfonic acid groups (63, 102). These substituted toxins have a lower specific toxicity or binding at the sodium channel than saxitoxin does, but they should not be overlooked for possible medical use.

Tetrodotoxin was originally found in the roe, ovaries, and liver of the puffer or globe fish (*Tetodonotidae*) caught in the western Pacific ocean and was at first believed to be exclusively produced by this fish. More recently it has also been found in various other animals including the California newt, octopus, and frog (234) and in marine bacteria (51, 199, 220, 235). Dinoflagellates have been proposed as the original source of the toxin in puffer fish, which acquire it through the food chain (234).

The action of tetrodotoxin is like that of saxitoxin in blocking the sodium channel of excitable membranes of nerve and muscle tissue. In fact, it has been shown that both tetrodotoxin and saxitoxin block the inward current of sodium ions at equally low concentrations of 10^{-7} to 10^{-9} M and occupy the same receptor sites at the sodium channel (63, 103). The basic structure of tetrodotoxin is markedly different from that of saxitoxin and is chemically described as aminoperhydroquinazoline, with a molecular weight of 319. Although the two toxins are basically different in structure, they may be similarly classified as heterocyclic guanidines because of the guanidium group common to each toxin. Kao and Nishiyama (103) first proposed that the guanidium moieties of each toxin might enter at the sodium channel like guanidine and that the bulk of the remaining part of the molecule prevented the passage of the sodium ion.

Although this hypothesis may be consistent with many aspects of the action of the toxins, it appears that the chemical makeup of the molecule as well as the guanidine

group is involved. The reduction of the hydrated ketone group to an alcohol in the saxitoxin molecule completely destroys its effectiveness as a blocker of the sodium channel, and changes in the structure of tetrodotoxin also affect its binding (102). The purpose here is to point out in a general way the nature of the two toxins and how they might affect the action of other toxins used for treatment of hyperactive anesthetics. The fact that saxitoxin enhances the action of local anesthetics has raised some thoughts on the relation of one toxin to another on an excitable membrane. Reviews by Catterall (36), Kao et al. (102, 103), and Borison et al. (22) give detailed descriptions of the action of the microbial neurotoxins saxitoxin and tetrodotoxin (binding site 1 at the sodium channel) and compare them with neurotoxins from other nonmicrobial sources that affect the sodium channel, such as veratridine, aconitine, batrachotoxin, grayanotoxin, and the low-molecular-weight basic polypeptide toxins isolated from scorpion venoms, fish-hunting cone snails, and sea anemone nematocysts (binding site 2 at the sodium channel). These reviews point out the various mechanisms by which toxins might affect the nervous system via action at the sodium channel. From the proposed action it seems reasonable to assume that there may be value in the use of combined toxins for control of nervous activity.

It is quite interesting that the action potential at the sodium channel is also affected by certain substances, such as guanidine and 3,4-diaminopurine, that reverse or bypass the blocking action of botulinum toxin. Although these substances are not particularly good antidotes for the toxin, their action indicates a relationship between the action potential at the sodium channel and the liberation of a neurotransmitter at the nerve ending. One might assume, therefore, that substances such as saxitoxin, tetrodotoxin, or others that alter the action potential at the sodium channel should warrant further investigation for possible medical application. Guanidines, an effective substitute for sodium in action potential generation in excitable membranes (93), is also reported to relieve symptoms of botulism (39), suggesting that there may be interactions of toxins at the myoneural junction, a field that warrants further study.

Besides the microbial neurotoxins described thus far, there are other, less well understood microbial neurotoxins that may be found valuable for nerve and muscle control mainly because of their action at the sodium channel. *Gymnodinium breve*, a marine dinoflagellate responsible for the Florida red tides and the tremendous fish kills in that area, produces several toxins, two of which are neurotoxins designated brevetoxins A and B, that have some action at the sodium channel (22). These two toxins are lipid-soluble polyethers with a molecular weight around 900. Brevetoxin A has an indirect action on the sodium channel in that it enhances channel activity in the presence of toxins that bind to receptor site 2 at the sodium channel, but not to receptor site 1.

Gambierdiscus toxicus, a tropical reef-dwelling dinoflagellate, produces several toxins, including one designated ciguatera, which opens voltage-dependent sodium channels in cell membranes (186). This toxin is a lipid-soluble polyether with a molecular weight of 1112 and is concentrated as it is passed up the food chain to large predatory reef fish consumed by humans. The disease in humans affects both gastrointestinal and neurological systems. The neurological symptoms usually begin within 24 h and may last a month or more, indicating nerve blockage or damage requiring regeneration of nervous tissue. Afflicted persons experience cir-

cumoral paresthesias, paresthesias or paralysis of the extremities, and muscle pain.

Natural blooms of the freshwater blue-green alga (*Cyanobacterium*) *Aphanizomenon flos-aquae* which occur periodically in lakes of the northern United States and certain provinces of Canada have caused poisoning of farm animals from drinking the water. This organism produces several toxins including saxitoxin and anatoxin. Another species of this group, *Anabaena flos-aquae*, produces a substance that affects acetylcholine receptors in muscle membranes (35, 97). Some mycotoxins affect the nervous system in various ways. Scleramine, upon biological conversion to a quaternary amine, causes excessive salivation in farm animals and acts similarly to acetylcholine (30, 41). These organisms and other microorganisms produce other neural toxins, but little is known of their action and importance in pharmacology and physiology.

CONCLUSIONS

Botulinum toxin type A has been found useful for the treatment of many hyperactive muscle disorders by intramuscular injection, and the FDA has licensed the toxin for treatment of strabismus, blepharospasm, and hemifacial spasm. It is the first microbial toxin to be used for human treatment. Because it is injected into humans, purity is of prime importance and, therefore, during the production by culturing and purification, it must not be exposed to any substances that might be carried in trace amounts to the crystalline toxin and cause undue reactions in the patient. Injection of the toxin into muscle tissue has opened a new field of investigation into the action of the toxin on muscle and nerve tissue and has been beneficial to many humans who suffer from dystonias.

Types of botulinum toxin other than type A toxin and perhaps tetanus toxin may be useful for human treatment if patients develop immunity to type A toxin. Saxitoxin stimulates and prolongs the action of local anesthetics, suggesting the use of combined toxins for human treatment. Some microbiological toxins are described for possible use alone or combined with botulinum toxin for medical treatment.

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2

Structures of Botulinum Neurotoxin, Its Functional Domains, and Perspectives on the Crystalline Type A Toxin

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INTRODUCTION

It was 1895. In the village of Ellezelles, Belgium, 34 members of a music club had eaten some raw salted ham after performing at a funeral. Within the next 20 to 36 hours most of the musicians developed a neuromuscular syndrome—three died, and 10 others nearly died. Professor E. Van Ermengem isolated the culprit from the food and the victims of food poisoning and named it *Bacillus botulinus*; the anaerobic bacterium was later named *Clostridium botulinum* (1,2). The neurotoxic substance produced by the bacterium became known as botulinum toxin, a protein, and gained notoriety as the most poisonous poison (3). In the New World, 84 years after the discovery of the agent of deadly food poisoning, Dr. Allen B. Scott of San Francisco first reported how to use the most poisonous poison for therapeutic purposes in humans (4,5). Because of this pioneering breakthrough, lauded by Carl Lamanna (6) as "the most imaginative, technically brilliant, and courageous of such applied research," today more people in the United States are exposed to botulinum toxin through deliberate injection by clinicians (7) than by unknowingly ingesting it as a food poison (8).

The proverbial beating of swords into ploughshares was achieved with a body of knowledge mostly accumulated from the pharmacological studies of the toxin (primarily serotype A, see below), which in 1946 became available as a crystallizable preparation (9-11), and with sparse knowledge about the biochemistry of the neurotoxin (NT) (12) and its mechanism of action (13). Another remarkable aspect of this development is the low-grade purity of the NT preparation that was used in the early pharmacological studies (12-15), as well as in the pioneering therapeutic application, and is still being widely used.

Now we know that four primary sequential events lead to the manifestation of the well-known neurotoxicity of botulinum NT: (1) the NT binds to the cholinergic membrane at

the neuromuscular junctions, (2) the bound protein reaches inside the nerve endings through the endocytotic/lysosomal vesicle pathway, (3) a segment of the NT penetrates through the endosomal membrane into the cytosol, and then (4) this segment, acting as an enzyme, disrupts the neurotransmitter secretory machinery, resulting in blockage of acetylcholine release and consequent flaccid paralysis. The entire structure of the NT may be regarded in a simple model as a combination of three structural segments designated A-C-B, each with a functional role and joined linearly in that order. Segment B recognizes the NT-specific receptors and allows the NT to bind to the target cells. Segment C forms channels in the endosomal membrane and facilitates penetration of segment A (or A plus segments of C and B) into the cytosol. The enzymatically active segment A causes intracellular injury.

This chapter deals with two primary topics. First, the structural features of the NT and its three segments that now appear directly relevant to the NT's mechanism of action are discussed. A comprehensive review in this area is not presented. The second topic is a critique of the crystalline toxin type A, which contains no more than 20% by weight of the neurotoxin protein, the other 80% of the material being nonneurotoxic macromolecules (16,17) that are yet to be characterized; nevertheless, this is the preparation used clinically (18,19).

THE NEUROTOXIN

Sources of the Neurotoxin

The NT produced by the ubiquitous bacterium *C. botulinum* is found as seven antigenically distinguishable serotypes A, B, C, D, E, F, and G. Certain strains of *C. baratii* and *C. butyricum* have been identified within the past few years that produce NTs similar to classical botulinum NT serotypes F and E, respectively (2,20,21). A minor antigenic relatedness between types E and F has been known (22); with the advent of monoclonal antibodies a common epitope has been detected among types B, C, D, and E (23). One strain of the bacterium produces one serotype, but there may be exceptions to this (20,22). Serotype C at one time had two designations, C1 and C2. The C2 toxin, which ADP-ribosylates nonmuscle actin, is not the NT (2).

Structure of the Neurotoxin

Primary (Covalent) Structure

The neurotoxic protein is synthesized as a single-chain protein (mol. wt. ~150,000). Protease(s) endogenous to the bacterium cleave the single-chain protein within a narrow region inside a disulfide loop located about one-third of the way from the N-terminal to the C-terminal (Fig. 1a). This cleavage is called nicking. The proteolytically processed product—the dichain NT—now contains a light (L) chain (mol. wt. ~50,000) and a heavy (H) chain (mol. wt. ~100,000) that remain linked by an inter-chain disulfide or disulfides and noncovalent bonds. In the absence of the endogenous proteolytic enzymes the single-chain NT remains as such and after isolation from the bacterial culture can be nicked by the exogenous proteases (such as trypsin) into the dichain protein. The proteolytically processed dichain NT is more potent than the single-chain NT. The higher potency is evident whether the assay is mouse lethality, paralysis of neuromuscular junction preparations, or blockage of neurotransmitter release (24–27). This enhancement of biological activity is referred to as activation (28). The type A NT isolated from a 96-hr

incubated bacterial culture is found in the dichain form and fully activated (i.e., treatment with trypsin does not activate it further). The type E NT isolated from a 96-hr incubated culture is a single-chain protein, and following controlled trypsinization (nicking) the dichain NT exhibits more than 100-fold activation (12,28). The proteolytic processing at the nicking region involves cleavage of more than a single peptide bond; the result is excision of several amino acid residues. In the cases of type A and type E at least four residues (Gly-Tyr-Asn-Lys) and three residues (Gly-Ile-Arg), respectively, are removed (29,30), as depicted in Fig. 1a.

The complete amino acid sequences of NT serotypes A-F have been deduced on the basis of the corresponding nucleotide sequences (31,32 and references in Ref. 31). The predicted amino acid sequences indicate that the single-chain NTs are made of 1295 to 1251 amino acid residues; they are schematically represented in Fig. 2a. Among these six serotypes type E is the shortest (1251 residues) and type A is the longest (1295 residues). During proteolytic processing of the single-chain NT at the nicking region, additional cleavage at the NT's N-terminal (and hence excision of small peptides) does not occur. Direct amino acid sequence determinations of the NT types A, B, and E have demonstrated that their original N-terminals remain intact. Whether the original C-terminals of the single-chain NT remain in the proteolytically processed NT is yet to be determined.

Alignment of the amino acid sequences (not presented here, but see Refs. 31 and 32 for details) show an overall low homology (~50%), although several short stretches of varying lengths are homologous. This is understandable, because too much homology in sequence would endow the NT serotypes with common antigenic epitopes. Since these proteins do not show serological cross-reactivity (except as mentioned before), their primary and secondary structures are likely to be more dissimilar than similar. The similarity among them is likely to be just enough to conserve the structures required for similar functional properties such as receptor binding, channel formation, and intracellu-

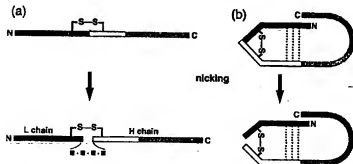


Figure 1 Proteolysis (nicking) converts the single-chain neurotoxin (NT) to dichain NT. Structure of the NT is represented in the straight line (panel a) and folded (panel b) configurations. Cleavages of more than one peptide bond during nicking are depicted by release of four amino acid residues. The light (L) and heavy (H) chains (mol. wt. ~50,000 and ~100,000, respectively) of the dichain (nicked) NT remain linked by a disulfide bond and noncovalent bonds (dotted lines between the L and H chain, see panel b). The two halves of the H chain, the N-terminal and C-terminal halves, are identified as the white and shaded segments, respectively.

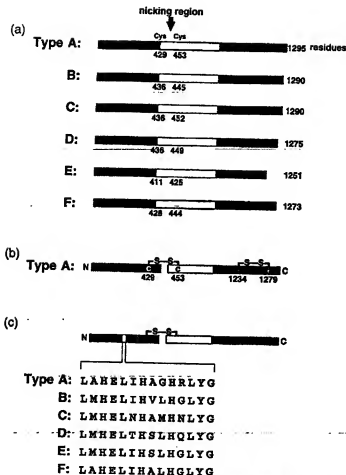


Figure 2 Covalent structures of the neurotoxin (NT) serotypes and a few of their important homologous segments. (a) Total number of amino acid residues, deduced from nucleotide sequences (31,32) present in the single chain NTs before proteolytic processing; also identified are the Cys residues that probably form the interchain disulfide in the dichain NTs. (b) Dichain type A NT and its Cys residues that have been deduced to form inter- and intrachain disulfides. (c) Homologous segment of the L chains of the NTs, containing the sequence His-Glu-*AXX-AXX*-His-*AXX-AXX*-His, and the zinc-binding motif.

lar inhibitory action (note below that even some of these properties of the NT serotypes are not identical).

... Two Cys residues, the locations of which are conserved among all serotypes and indicated by the residue numbers (e.g., 429 and 453 in type A), flank the nicking region (Fig. 2a). These two Cys residues in the single-chain protein probably form the intrachain disulfide. The length of this disulfide bridge varies from 23 residues (in type A) to 8 residues (in type B). This intrachain disulfide, following nicking of the single-chain NT to the dichain NT, presumably acts as the interchain disulfide between the L and H chain. Evidence for this is inferential; direct chemical proof is yet to come. An intrachain disulfide near the C-terminal of types A and B has been also deduced (see reference in Ref. 33). In the case of Type A, Cys 1234 and Cys 1279 form this intrachain disulfide, as shown in Fig. 2b (33). Type B and E NTs have Cys residues in equivalent locations (31).

Roles of Cys Residues in Biological Activity

Long before the exact number of Cys residues present in type A NT became known (from its amino acid composition and sequences), their importance in the protein's neurotoxicity as free -SH groups and participants of disulfide bond(s) was of obvious interest. On the basis of the simple mouse lethality assay, the free -SH groups were found not essential for toxicity (34), but the integrity of the disulfide bond(s) was found essential (35). These early conclusions have recently been confirmed and refined in the context of the distinct steps of the NT's mechanism of action and the activities of the three functional domains of the NT. It is now known (1) that the productive binding of type A NT with the receptors does not depend on the disulfide bonds (inter- and intrachain) and free -SH groups; (2) that internalization (of the L chain) depends on the integrity of one or both disulfides (experiments could not distinguish between the inter- and intrachains, since selective reductive cleavage of one was not possible); (3) that the L chain's intracellular inhibitory activity does not require a free -SH group (because the L chain was active following alkylation of the free -SH) (36); and (4) that removal of the 32 C-terminal residues of the L chain (which includes Cys 429) does not diminish its intracellular activity (37). Points 2 and 4 together indicate (1) that disulfide bond(s) linking L and H chains, and the Cys 429 on the L chain (see Fig. 2b), have no role in the actual intracellular inhibitory action of the L chain, and (2) that the roles of the interchain disulfide (between Cys 429 and Cys 453) and the participant Cys 429 are to maintain a covalent linkage between the L chain and H chain, after nicking of the single-chain protein—otherwise the L chain, being easily separable from the H chain, might not reach the target cell interior (see below, Fig. 4). These deductions are consistent with the observation that the dichain NT presented to the cytosol of permeabilized cells, bypassing the steps of receptor binding and internalization (by endosomes), shows higher intracellular inhibitory activity after reduction of the interchain disulfide between the L and H chains (27); reduction presumably allows them to separate.

The Zinc-Binding Site of the Neurotoxin

A segment around the midsection of the L chain of all six NT serotypes sequences (Fig. 2c) contains the sequence His-Glu-Axx-Axx-His, which is the zinc-binding motif of Zn endopeptidases. The three proteases thermolysin, *Bacillus cereus* neutral protease, and *Pseudomonas aeruginosa* elastase contain Zn, and their three-dimensional structures, determined by x-ray crystallography, indicate that in each case an atom of Zn^{2+} is bound by a tetrahedral coordination with the two His of the motif His-Glu-Axx-Axx-His, while

the Glu residue binds a water molecule acting as the third Zn^{2+} ligand. The fourth ligand is another Glu residue (see Ref. 38 for further references). On the basis of this clue, atomic absorption analysis of type A, B, and E NTs were made; all three NTs contain one Zn atom per molecule of NT (mol. wt. $\sim 150,000$). Measurements of Co, Cu, Fe, Mn, and Ni were also made, and none was detected. Additional experiments demonstrated that this peptide segment binds Zn and that two His residues are involved in Zn coordination (38). The possibility that the NTs could be Zn endopeptidases was tested, and proteolytic activity has been found (see below, Functional Domains).

Secondary Structures

Analysis of the secondary structural elements (α -helix, β -sheet, β -turn, and random coil) of the NT types A, B, and E revealed that these proteins have highly ordered structures that are dominated by the β -sheets. About 62–72% of the amino acid residues are in the ordered structure at pH 7.2 (21–28% in α -helices plus 41–44% in β sheets), and the rest are in random coils (39). At pH 5.5, which approximates the pH inside the endosomes, the type A NT also retains the highly ordered structure: α -helix 29%, β -sheet 45–49%, and random coils 22–26% (40). The 29% α -helix content of the NT at pH 5.5 compared with 21% at pH 7.2 may reflect the acid-induced conformational change the NT presumably undergoes inside the endosome before channel formation (see below). Analysis of the L and H chains of type A NT following their chromatographic separation gave an interesting insight into their conformational stability (41). The sums of the α -helix, β -sheet, β -turn, and random coil contents of the separated L and H chain, as a weighted mean, were similar to the content of the corresponding structural elements in the NT (see Table 1), e.g., the sum of the α -helix content of the L chain (22%) and the H chain (18.7%), 19.8% as a weighted mean, was similar to the α -helix content of the NT (20%). In other words, the secondary structures of the L and H chains do not change significantly when they are separated. This stability indicates that the two chains, by virtue of their structural integrity, may express their individual biological activities even when physically separated. This notion agrees with two independent experimental observations: (1) The two chains can be separated and then recombined to form disulfide-linked NT (mol. wt. 150,000), which is highly active (see references, in Ref. 28). (2) The separated chains, although nonlethal by themselves, are biologically active, i.e., the isolated H chain forms channels in lipid bilayer membranes and binds to the receptor; the isolated L chain presented to the interior of a neuronal or chromaffin cell inhibits secretion of neurotransmitter (see references in Ref. 42).

Table 1 Secondary Structure Elements of Type A Neurotoxin and Its Light (L) and Heavy (H) Chain After Separation

| Protein | α -Helix | β -Sheet | β -Turn | Random coil |
|--|-----------------|----------------|---------------|-------------|
| Neurotoxin (mol. wt. $\sim 150,000$) | 20.0 | 37.5 | 15.2 | 27.2 |
| L chain (mol. wt. $\sim 50,000$) | 22.0 | 27.5 | 18.7 | 31.7 |
| H chain (mol. wt. $\sim 100,000$) | 18.7 | 40.0 | 13.0 | 28.2 |
| Sum of L and H chain (weighted mean) ^a | 19.8 | 35.8 | 14.9 | 29.4 |

These secondary structure elements were obtained from circular dichroism spectra (240–200 nm) of the protein (0.1–0.3 mg/ml) in 10 mM sodium phosphate buffer, pH 8.1, containing 100 mM NaCl, at 23–25°C.

^aCalculated as $(1 \times L \text{ chain} + 2 \times H \text{ chain})/3$ because the H chain is twice the size of the L chain.

Tertiary and Quaternary Structures

A simple model of the folded configuration of the NT has been built based on the results of limited proteolysis (33). Figure 3, which incorporates the ideas presented in Fig. 1b, shows the common narrow regions of the NT serotypes A, B, and E cleaved by various proteases (33). Three of these regions, sites 1, 3, and 4, are highly susceptible. Site 1, the nicking region, is about one-third of the distance from the N-terminus; site 3 is approximately at the middle of the H chain, and site 4 is near the C-terminus. Figure 1b has depicted (1) that following cleavage at site 1, the L chain remains bound to the N-terminal half of the H chain by disulfide and noncovalent bonds (dotted lines between the L and H chains); (2) that noncovalent interactions between the L chain and the C-terminal half of the H chain are virtually absent or extremely weak; and (3) that association between the two halves of the H chain is also absent or very weak. Thus cleavage at site 3 allows the C-terminal half (mol. wt. $\sim 50,000$) to separate from the rest of the molecule (mol. wt. $\sim 100,000$) very easily (see references in Ref. 33). The C-terminal half of the H chain (result of cleavage at site 3) has been found completely or extensively digestible by trypsin, chymotrypsin, and pepsin. The N-terminal half of the H chain, on the other hand, survives proteolysis remarkably (reviewed in Ref. 33). Proteolytic digestions of NTs carried out in various laboratories have not reported significant cleavage of the L chain. This suggests (1) that the L chain and the N-terminal half of the H chain probably are individually resistant to proteolysis and/or (2) that the association between these two segments of the NT makes their protease-susceptible sites unavailable for cleavage.

Many observations indicate that the natural foldings of the polypeptides protect the proteins from proteolytic assaults, that limited proteolysis of native proteins is usually restricted to interdomain regions, and that these susceptible regions are flexible hinges on the protein surface. Consistent with this view are the proteolysis-susceptible regions on the NT detected so far. The two halves of the H chain (generated by cleavage at site 3), the N-terminal and C-terminal halves, also retain after separation their channel-forming (43,44) and ganglioside-binding activities (45), respectively. Earlier it was noted that the L chain separated from the H chain retains biological activity. Thus, the three segments of the NT that exhibit biological function after separation, the L chain and the two halves of the H chain, each of $\sim 50,000$ mol. wt., represent three domains spaced in the polypeptide backbone by two hinges (indicated in Fig. 3b) and proteolytically susceptible regions. (These segments were referred to as A-C-B above, "Introduction".)

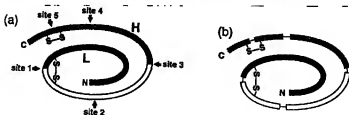


Figure 3 Model of folded configuration of the neurotoxin (NT) based on proteolytic digestions. (a) Sites on NT types A, B, and E cleaved by various proteases (reviewed in Ref. 33). (b) Flexible hinges (the proteolytically susceptible regions) of the NTs, spaced in the polypeptide backbone, are indicated as wasp-waist segments.

The above observations allowed us to build the model in Fig. 3 (33) the salient features of which are as follows: (1) sites 1, 2, 3, and 4 are on the surface and hence accessible to proteolysis, (2) most of the L chain is shielded by the H chain from proteolysis, (3) most of the N-terminal half of the H chain is closely associated with the L chain, (4) the C-terminal half of the H chain is not associated with the rest of the molecule, and (5) the C-terminal segment is highly accessible to receptors on the target cells (see below) and proteolysis, and it allows the NT molecules to associate to form what appear to be "dimers" (see below). The shape of the NT molecule is ellipsoid rather than spherical, so that one of its axes is longer than the others (the reasons are discussed in Ref. 33). The diameters of the NT serotypes A, B, and E, measured by dynamical light scattering, are 100 ± 4 , 110 ± 4 , and 100 ± 4 Å (46). The diameter of the type A NT, 96 Å (Stokes radius = 48 Å), measured many years ago by gel filtration (17), agrees well with the new data. It is not clear yet if this diameter represents the size of the NT monomer (mol. wt. 150,000) or dimer (mol. wt. $2 \times 150,000$).

Three independent lines of evidence indicate that the NT molecules associate to form entities larger than 150,000 (mol. wt. 300,000 and larger): (1) Chromatography of pure type A NT yielded type A NT molecules larger than 150,000 (47). (2) Polyacrylamide gel electrophoresis (without SDS) of pure type A NT demonstrated protein species of mol. wt. 300,000 and larger (33,43,48). The NT without the C-terminal half of the H chain (i.e., after cleavage at site 3) does not associate to form larger-molecular-weight species. (3) Crystals of pure type A NT also indicate dimerization (49).

The pure type of A NT (mol. wt. 150,000) has been crystallized in three different crystal morphologies; all three have the same crystal form and diffract to 3 Å (49). Determination of the three-dimensional structure of botulinum NT at atomic resolution now appears an achievable goal.

Functional Domains

The absolute neurospecificity and extremely high potency of the NT are attributable to its high affinity for specific receptors on the presynaptic membranes and to an enzymatic action, functions of the H and L chains of the NT, respectively (Fig. 4). To explain how the NT at extremely low concentrations can bind specifically to the nerve cells, the proposal of Montecucco (50) deserves reiteration. The NT first binds to the ganglioside-rich lipid membrane, then the lipid-NT complex moves laterally to reach and bind the NT-specific receptor, which is protease-sensitive. Accordingly, any docking of the NT molecule on the membrane results (following the "catch-and-delivery effect") in a productive binding with the NT-specific receptor protein. The lipid-binding step "is actually equivalent to concentrating the NT and its protein receptor in a much smaller volume . . . because the partners of the binding reaction are now restricted to the two-dimensional plane of the plasma membrane rather than in the three-dimensional water phase" (50). The two-step hypothesis agrees with experimental results. Two-dimensional, ordered arrays of NT types A and B form at the interface of a NT solution and phospholipid monolayer containing the ganglioside GT_{1b} . The NT binds the hydrophilic moiety of the ganglioside, and two-dimensional diffusion allows crystals to form (51).

Receptors (also referred to as acceptors) of high affinity have been identified (the K_D values, for example, for type A, B, and E NT range from 0.5 nM to 100 pM; see Refs. 2,15,42 for review). In fact, two receptor species, one with higher affinity and low populations, and another with lower affinity and higher populations, have been identified for type B NT (52). Some NT serotypes do and some do not share the same receptors;

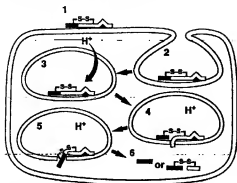


Figure 4 A simple diagrammatic representation of the sequential events that lead to the blockade of neurotransmitter release by the neurotoxin (NT). The NT binds to the receptor (black triangle) on the presynaptic membrane (step 1) via its heavy (H) chain. Endocytosis (step 2) internalizes the NT, which is now inside the endosome (step 3). The acidic pH inside the endosome (steps 3,4) induces formation of channels in the endosomal membrane by a segment of the H chain (step 4) that allows the light (L) chain to egress from the vesicle (step 5) into the cytosol (step 6), where the L chain acts enzymatically and disrupts the neurotransmitter secretion. This model accommodates the possibility that the single-chain NT, after binding the receptor and internalization, is nicked to the dichain by the endosomal proteases (between steps 3 and 4). The model does not attempt to define the step at which the disulfide bond between the L and H chain is broken.

e.g., types A and E share the same receptor, types A and B do not (53). The densities of the distinct populations of receptor/acceptor (mouse diaphragm) for NT types A and B have been determined; their average numbers are 153 ± 21 and 637 ± 131 per μm^2 of membrane, respectively (54). Black and Dolly (54) considered these densities too high relative to the number of NT molecules needed to induce *in vivo* paralysis (1 mouse LD_{50} being equivalent to $\sim 1.2 \times 10^{-11}$ g or 8×10^{-17} mol or 5×10^7 molecules of type A NT; at the maximum, 100–1000 molecules are needed to block neurotransmission at each synapse). Thus the receptor/acceptor densities greatly exceed the number of NT molecules needed to block a synaptic transmission, arguing against a "one-hit" model for the NT's mechanism of action. The role of high densities of acceptors therefore appears to be to concentrate the NT on the target membrane surface for the next step—delivery inside the cell via intracellular vesicular compartments.

The ganglioside (GT_{b})-binding site on type A NT is primarily confined within the C-terminal (50,000 mol. wt.) half of the H chain; the N-terminal half of the H chain did not bind GT_{b} under any conditions tested, and the L chain exhibited a small degree of binding, which might be nonspecific (45). The demonstrated ganglioside-binding site on type A perhaps can be generalized for the other serotypes. Analogy with tetanus NT, which is very similar to botulinum NT in structure, structure-function relationship, and mechanism of action (2,15,55), further indicates the receptor-binding role of the C-terminal half of the H chain. A protein (mol. wt. $\sim 20,000$) expressed by PC12 cells following differentiation with nerve growth factor binds tetanus NT, and the binding is neuro-

specific. Only the C-terminal half of the H chain of tetanus NT binds to this protein; the remaining segment of the NT, i.e., the L chain and the N-terminal half of the H chain (mol. wt. $\sim 100,000$), did not bind (56 & reference 33 therein). These experiments once again demonstrate the role of the C-terminal half of the H chain in receptor binding. A protein(s) that appears to act as the receptor protein for botulinum NT type B has been reported (52,54).

According to the currently held view, when the pH inside the endosomes drops, the NT entrapped within this acidic environment undergoes some conformational change leading to an insertion of a segment of the NT into and across the endosomal membrane (Fig. 4, step 4). This poorly understood process somehow allows the L chain or the L chain and a segment of the H chain to cross the membrane and reach the cytosol. The experimental evidence behind this scenario is low pH-induced channels in lipid bilayers formed by the H chain and the N-terminal half of the H chain. Neurotoxin added to one side of an artificial lipid membrane forms few channels when on both sides the pH is ~ 7.0 or ~ 5.0 ; however, when the pH on the side of the NT is lowered to ~ 5.0 and kept near 7.0 on the other side, many channels are formed. The channel-forming activity is confined to the N-terminal half (mol. wt. $\sim 50,000$) of the H chain (43,44, and references therein). This pH gradient favorable to channel formation mimics the condition of low pH inside the endosome and physiological pH of the cytosol, i.e., outside the endosome. A narrow segment of the N-terminal half of the H chain of type A NT, residues 650-681, has been located that appears to be responsible for channel formation (57). Whether these channels provide a large enough opening for a polypeptide to pass through is not yet known.

Enzymatic activity of NT was recently demonstrated based on the proteolytic cleavage of a neuronal protein (58). Incubation of highly purified small synaptic vesicles (rat cerebral cortex) with the NT serotype B cleaved a single peptide bond (between residues 76 and 77, Gln-Phe) of synaptobrevin-2 (also called VAMP), which is a synaptic vesicle-associated integral membrane protein. Of the two isomers of synaptobrevin, synaptobrevin-1 has Val in the position of Gln; the Val-Phe bond in synaptobrevin-1 was not cleaved. The L chain of tetanus NT also cleaved synaptobrevin-2, and thus by analogy the proteolytic activity of botulinum NT is confined in its L chain. Unlike the type B NT, type A and type E NTs did not show any cleavage (58).

The nonidentical actions of NT types A, B, and E are not surprising; the intracellular inhibitory effects of the type A, B, and E NTs studied in permeabilized chromaffin and PC12 cells also show notable differences. The Ca^{2+} -stimulated secretion of norepinephrine was inhibited most by type E and least by type A (26,27). Long before permeabilized secretory cells were utilized to study the intracellular inhibitory actions of NT, Sellin (53) had proposed, on the basis of other experimental evidence, that various NT serotypes do not follow a single mechanism of action, and that the intracellular site of action of type A is distinct from those of types B, E, and F. (See Note Added in Proof.)

Potential Use of Different Neurotoxin Serotypes and Chimeric Neurotoxins

The different NT serotypes could be used clinically to exploit their nonidentical pharmacological actions rather than only to obviate the immunity that may develop from repeated administration of a single serotype. This idea must have crossed many minds. Further additions to the repertoire of pharmacological differences may be made by generating chimeric neurotoxic molecules, e.g., an NT made of L chain of type E and H chain of type A. The following considerations of the structures and structure-function relationships of the NTs favor the above two ideas. The different paralytic effects (magnitude, rapidity, duration, and recovery/reversal) produced in identical neuromuscu-

lar preparations by different NT serotypes (e.g., type A vs. type E, in Refs. 59,60) are probably rooted in the intrinsic structural features of the functional domains of the NT (42) and some of the components of the target neuronal cells. The population of receptors specific for the different NT serotypes present on the neuromuscular junctions of various muscles may not be identical. A therapeutic target area X may be significantly richer in receptors for NT type A than for types B and E, and other target areas Y and Z may have more receptors for NT type B and E, respectively. Experimental knowledge of such differences would indicate that better tools to paralyze muscles at target areas X, Y, and Z could be NT types A, B, and E, respectively. This consideration, based on the function of the H chain, attempts more efficient capturing of the NT and delivery of it inside the target neuronal cells. A corollary of this approach is that a lower amount of administered NT protein also lowers the immunogen load. The actual inhibition of neurotransmitter release could be further manipulated by presenting the target cells' cytosol with an L chain, chosen on the basis of its intracellular inhibitory activity. Thus a chimeric NT can be designed and made from L and H chains from two different NT serotypes, each chosen on the basis of its functional properties. Certain combinations of these two structures could provide therapeutic agents more suitable than the NTs we know of now.

Production of chimeric NT is clearly feasible. The L and H chains of NTs after separation can be recombined to generate neurotoxic dichain NT (mol. wt. ~150,000, see references 4-6 in Ref. 61). The chemistry involved in this approach has allowed generation of type A NT that was selectively radiolabeled at either the L or the H chain (one of the separated chains was radiolabeled and then combined with the corresponding nonlabeled chain) (61). More convincing is that the L and H chains of tetanus NT have been combined with H and L chains of botulinum type A NT, and that the chimeric NTs (part tetanus, part botulinum) exhibited predicted biological activities (62,63).

CRYSTALLINE TYPE A TOXIN

The preparation of type A NT that has found rapid and wide acceptance for therapeutic use is the crystallized mixture of type A NT and nonneurotoxic proteins; since 1967 (64) this complex material has hardly received a rigorous analytical scrutiny satisfactory to the standards of modern protein biochemistry. Crystallographic data for the preparation have never been reported.

The following properties of the crystallized toxic preparation are used to judge its purity (19):

1. Around neutral pH it absorbs maximally at 278 nm.
2. The ratio of its absorbance at 260 and 278 nm is 0.6 to 0.55.
3. An absorbance of 1.65 at A_{278} is equivalent to 1 mg protein/ml.
4. Its isoelectric point is 5.6, and at acidic pH it migrates in electric field as a single band.
5. The nitrogen content is 16.2%.*
6. It contains about 0.1% or less RNA.
7. Its specific toxicity is $3 \times 10^7 \pm 20\%$ LD₅₀/mg protein.

The properties listed as items 1, 2, 3, and 5 are not unique features of this protein preparation because these could be parts of general properties of proteins.

*This is old data (65), although "the nitrogen content of the toxin was redetermined and found to be 16.08%" (64).

Comments on the Optical Properties

The three aromatic amino acids tryptophan, tyrosine, and phenylalanine, in aqueous solution, absorb light at wavelengths of 250–300 nm in characteristic fashions. Tryptophan, tyrosine, and phenylalanine maximally absorb near 280, 275, and 260 nm, respectively. Thus the absorption profile of a protein in aqueous solution, in the region 250–300 nm, is determined both qualitatively and quantitatively by the absolute number of the three aromatic amino acids and their relative proportion. A protein completely free of nucleic acid (which absorbs maximally near 260 nm) can have significant absorption at 280 and 260 nm, the relative extent of which depends on the characteristic amino acid composition.

The absorption profile of pure type A NT (Fig. 5) shows an absorption maximum at 278.2 nm, a minimum at 249.5 nm, and a steep rise below 250 nm. (Proteins, like many organic compounds, also extensively absorb below 250 nm.) The pure type B and E NTs, prepared in our laboratory, exhibited 278.0 and 277.7 nm as absorption maxima and 249.7 and 250.0 nm as absorption minima, respectively (Table 2). The ratios of the absorption maxima and minima for these type A, B, and E NTs were 0.301, 0.301, and

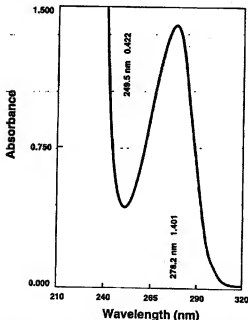


Figure 5 Absorption profile of pure type A neurotoxin (mol. wt. 150,000) in 0.1 M sodium phosphate buffer, pH 7.4, plus 0.05 M NaCl (UVIKON 860 Spectrophotometer, Kontron Instruments).

0.286, respectively. The ratios at A_{260}/A_{278} of the NTs purified by chromatography are invariably below 0.5.

The theoretical molar extinction coefficient of type A NT (mol. wt. 150,000) at 280 nm, calculated from the molar extinction coefficients of tryptophan, tyrosine, and phenylalanine and their known abundance in the protein (15, 74, and 70 residues, respectively, see ref. 3 in 31) is 181,150, which means that 1 mg/ml of the NT has an absorbance of 1.212 at 280 nm (B. R. DasGupta, unpublished data). The large difference between the deduced absorbance of the pure NT, 1.21, and that of the crystalline complex, 1.65, is probably due to the nonneurotoxic components in the complex.

The notion that "the purity of the crystalline toxin cannot be defined strictly in terms of percent purity because of small amounts of undefined material absorbant at 260 nm, most probably nucleic acid material" (19) is not completely right, because as mentioned above any protein with aromatic amino acid residue absorbs at 260 nm even if nucleic acid is entirely absent. Thus the use of the ratio of absorption at 260 and 278 nm has very limited value in judging whether a protein is free from other proteins and nucleic acid. This point is also articulated by the data in the last column in Table 2. Note that any mixture of pure type A, B, and E NT, in any proportion, will have a ratio of absorbance of ~0.50 at 260 and 278 nm.

Electrophoretic Analysis and Criteria for Testing Purity

The electrophoretic migration of the crystallized type A toxin preparation as a single band at acidic pH, an observed fact, has questionable value for judging the purity of the protein preparation. Although it has been known since 1980 (66) that the nonneurotoxic components that form a complex with type A NT can be resolved electrophoretically in the presence of SDS into at least 7 proteins, some of which were partially sequenced recently (67), this author is not aware that the crystalline preparation has been defined as to the exact number of proteins it is composed of and their relative proportion. The basis of the idea that the crystalline toxin (mol. wt. 900,000) "is composed of two molecules of neurotoxin (ca. 150,000 M), non-covalently bound to non-toxic proteins" (19) was not described and is not known.

Consideration of the purity of a substance requires a definition of the substance; the more exact the definition of its composition, the more meaningful consideration of its purity becomes. Many biologically active proteins composed of multi-subunits that are homomers and/or heteromers (a few examples are ribosome, ATPase, acetylcholine receptor, hemoglobin, pertussis toxin, and cholera toxin) have been precisely defined.

Table 2 The Ultraviolet Absorption Properties of Botulinum Type A, B and E Neurotoxins

| Neurotoxin | Absorption | | Ratio of absorption | |
|------------|-------------|-------------|---------------------|---------------|
| | Minimum (a) | Maximum (b) | at a/b | at 260/278 nm |
| Type A | 249.5 nm | 278.2 nm | 0.301 | 0.491 |
| Type B | 249.7 | 278.0 | 0.301 | 0.495 |
| Type E | 250.0 | 277.7 | 0.286 | 0.468 |

Type A NT in 0.1 M Na-phosphate buffer pH 7.4, plus 0.05 M NaCl.

Type B NT in 0.1 M Na-phosphate buffer pH 8.0, plus 0.05 M NaCl.

Type E NT in 0.1 M Na-phosphate buffer pH 7.4, plus 0.01 M NaCl.

The published literature does not inform us exactly how many different proteins (and nucleic acids) combine in what relative proportion with the NT to form the complex that is eventually crystallized. This understanding needs to be developed rigorously; before the issue of purity of the crystallizable complex can become meaningful. If, for example, it can be shown that the crystallized complex is made of NT and, let us say, seven different nonneurotoxic proteins, and all eight proteins combine in certain fixed relative proportion (e.g., 1:1:1:1:1:1:1), then the purity of the crystallized complex can be qualified according to the presence of anything in addition to the NT and the hypothetical seven other proteins.

Simple experimental techniques are available to develop this information objectively. Electrophoresis of the complexes of NT and nonneurotoxic proteins in polyacrylamide gel in the presence of SDS resolves the complexes into multiple bands that can be visualized after staining with Coomassie blue or silver (see Ref. 67 for such patterns from type A, B, and E complexes). The total number of different proteins present in a complex and their molecular weights can thus be delineated. Densitometric scanning of the band patterns in such gels provides a dependable quantitative profile of the protein components and thus of their relative proportion in the complex.

Toxin Complex and Crystalline Toxin A, History and Current Status

In the bacterial culture, the NT exists as a large complex made of the NT (mol. wt. ~150,000) and nonneurotoxic protein(s); noncovalent association keeps the proteins together (Fig. 6). The nonneurotoxic protein(s), which seems to be produced by the bacteria simultaneously with the NT, may or may not agglutinate red blood cells, i.e., may or may not have hemagglutinating (Hn) activity. Based on this property these nonneurotoxic proteins have been designated Hn⁺ and Hn⁻ (67).

Attempts made in the mid-1940s to purify the NT from the bacterial culture resulted in the isolation of a complex of the NT and nonneurotoxic proteins, which were crystallized in 1946 by two groups: Lamanna, McElroy, and Eklund (9) and Abrams, Kegeles, and Hottel (11). The molecular weight of this complex is 900,000. Duff et al. (68) modified the earlier protocols for partial purification of the NT (9,10,11) and also obtained a complex that crystallized. This modified protocol, developed in 1957 (68), was used to prepare the crystallized mixture of type A NT and nonneurotoxic proteins that was introduced for therapeutic use (18,19). The mixture of NT, other proteins, and nucleic acid that crystallizes readily was obtained entirely by differential precipitation steps (9,10,11,68) and without the benefit of high resolution achieved by chromatography. Anion-exchange chromatography of the crystalline type A toxin separated the NT from the nonneurotoxic proteins; only about 20% of the protein in the crystalline preparation was found in the NT (16,17).

Thus the preparation may well be called crystalline hemagglutinin rather than crystalline toxin. The alternative name, perhaps frivolous, is more apt simply because the weight of the argument is against the NT content in the preparation. Referring to the crystalline type A botulinum toxin, Lowenthal and Lamanna (69) wrote that "work on the characterization of the type A botulinum toxin was in reality characterization of the toxin-hemagglutinin complex, rather than of toxin alone." The preparation of type C toxin complex that was crystallized (70,71) contains hemagglutination activity, and polyacrylamide gel electrophoresis (without SDS) revealed that it is a mixture of at least three proteins (70).

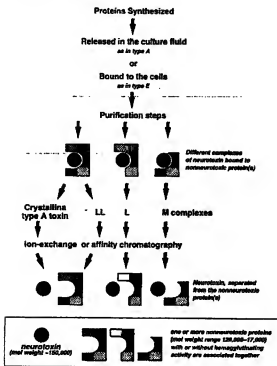


Figure 6 Schematic description of formation of complexes between the neurotoxin (mol. wt. ~150,000) and nonneurotoxic proteins and their eventual separation by chromatography.

The protocol in use since 1957 for isolating type A toxin complex as crystals (68) was modified in 1977 (72). In this improved protocol, one chromatography step was introduced that substituted precipitation of the toxin complex by ethyl alcohol. There were three beneficial results: (1) a twofold greater recovery (33% vs. 17%) of the total toxicity present in the bacterial culture was gained; (2) more effective removals of pigments and nucleic acids (the A_{260}/A_{278} ratio was 0.52) were achieved; and (3) the crystal lots produced were of greater uniformity.

Crystals of the mixture of proteins produced according to the improved procedure (72) and by following Duff et al. (68) contained three antigens (detected by double-diffusion serology with anti-crystalline serum), one of which was the NT (detected by anti-pure NT serum). The minor antigen was eliminated from the crystals produced by either method following recrystallization (72).

Sugii and Sakaguchi (73) had concluded earlier that "the generally accepted notion that type A crystalline toxin consists of two components, neurotoxin and hemagglutinin,

will have to be changed." They isolated, using chromatography, two kinds of complexes, called L and M (with specific toxicity of $2.5-3.0 \times 10^4$ and $4.5-5.0 \times 10^4$ mouse MLD/mg N₂, respectively). Both were composed of at least three distinct components, NT, hemagglutinin, and an inert protein. The nonneurotoxic component of M complex contained little or no hemagglutination activity. The nonneurotoxic materials in crystallized toxin and L complex consisted of two distinct antigens and were antigenically identical. The inference was that L complex and crystallized toxin are similar in composition.

The two reports (72,73) indicate that methods using chromatography are available to isolate complexes of NT and nonneurotoxic proteins that in purity are at least equivalent to or better than the crystallizable product produced following the protocol of Duff et al. (68).

Type B NT was also initially isolated as a complex of NT and nonneurotoxic proteins (74); the molecular weight of the large complex was found to be 500,000 (75). Modification of this purification protocol that did not include any chromatographic step yielded a preparation that appeared as a single entity and a large complex (the S_{20W} value was 12.7) by ultracentrifuge analysis (76). Only ion-exchange chromatography could resolve the complex into the NT (initially reported as 165,000 mol. wt.) and the nonneurotoxic proteins (77,78).

Neurotoxin serotypes A and B do not serologically cross-react. Some of the nonneurotoxic proteins associated with type A and type B NTs do cross-react. This has produced a great deal of false data and confusion. Antiserum produced against the complex of type A does not neutralize type B NT (and vice versa) when the assay is mouse lethality. However, the same polyclonal antiserum (i.e., produced against the complex of type A) shows positive reactions in Ouchterlony, ELISA, and RIA assays against a type B NT preparation if it has a detectable amount of the nonneurotoxic Hn⁺, Hn⁻ proteins. These problems can be avoided by producing the polyclonal antiserum against the pure NT completely free of the associated nonneurotoxic proteins. Cocrystallization of two or more substances (organic or inorganic) is a common phenomenon, and it is not peculiar to proteins. Repeated recrystallization of such a mixture using the same or different solvents often generates purer crystallized material, generally at the cost of yield, but also often produces a mixture whose composition remains constant, and thus further selective enrichment of one component cannot be attained by the same approach (azeotropic mixtures and eutectic compositions present analogous situations).

Attempts to improve the quality of the material in the crystallized complex of type A NT by recrystallization appear to have reached the point of diminishing returns. This was recognized as early as 1946, when Lamanna and colleagues (10) wrote: "Amorphous, crystalline, and recrystallized materials have been determined to have the same toxicity within the limits of errors of mouse titration." When the ratio of absorption at 260 and 278 nm was used as a parameter of purity (and as an indicator of the presence of nucleic acid) it was close to 0.6 and near 0.55 at the end of the first and second recrystallization steps, respectively. The third crystallization reduced the ratio only slightly, with a considerable loss of the material (19). The purity of the crystallizable proteins is likely to improve if separation techniques different from the ones currently used (19,68) are employed to break out of the "constant composition deadlock." Introduction of a simple chromatographic step, as mentioned above (72), in the old protocol (68) of preparation of the type A NT complex has indeed reduced the A_{260}/A_{278} ratio to 0.52 and increased the yield of the final product by a factor of 2.

Detoxification of Crystalline Type A Toxin and pH

A recent carefully conducted study (79) found that the freeze-dried crystalline type A toxin (used clinically), which is stable for months under refrigeration, was 43.9% detoxified (statistically significant) after reconstitution for clinical use (50 ng toxin, 500 μ g human serum albumin, and 900 μ g NaCl at pH 7.3) and storage at 6°C for 12 hr but was not detoxified for the first 6 hr. The authors of this study, noting that the toxin preparation is more stable in solution at pH 6.2 than at pH 7.3, suggested, justifiably, that the reconstituted toxin be maintained at pH 6.2 rather than at pH 7.3. The time-dependent inactivation of the toxin preparations above pH 7.0 was observed by the early workers* and probably for this reason, in many of the past studies with crystalline type A toxin the pH was maintained strictly below 7.0.

One likely reason for the pH- and time-dependent inactivation of the toxin preparations is the possible presence of traces of protease(s) whose activity is suppressed at acidic pH and is high near pH 7.3. This reasoning is favored by the following considerations: (1) The pH- and time-dependent inactivations of toxicity were observed primarily when the neurotoxic preparations were not chromatographically purified. (2) After chromatographic purification, the type A complex could be exposed to pH above 7.0 without notable loss of toxicity (73,80). (3) The pure NT is stable for months at pH 7.9 and 4°C (81). The postulated pH-dependent inactivating agent, probably a protease, therefore appears to be separable from the NT if chromatographic steps are employed.

The rationale for clinical use of the impure type A NT (about 80% of protein in the crystallized complex is nonneurotoxic protein) is that the nonneurotoxic proteins "bound to the neurotoxin apparently play an important role in maintaining the toxic shape of the neurotoxin" (19). If this is so, why then does the diluted crystallized preparation need to be fortified by adding gelatin or serum albumin to maintain neurotoxicity, as is commonly done (18,19)? Does this suggest that the pure NT (mol. wt. ~150,000), separated from the nonneurotoxic proteins, may also be fortified from detoxification with some other clinically acceptable stabilizing agent(s)? Such a preparation concocted with a known concentration of the pure NT and a defined added entity (for storage) could have a precisely determined composition.

Progress in this direction is not evident from publications, perhaps because of the assertion that "an important point regarding the use of purified neurotoxin besides its instability is the fact that it cannot be prepared with constant composition and activity" (18). This is not true, and this prevailing view needs rectification. The NT (mol. wt. ~150,000) isolated from the complex by ion-exchange chromatography, first reported in 1966 (16) and now routinely prepared in various laboratories (73,48), has stable activity;

* (a) "The toxin . . . is extremely sensitive to pH values above 6.5 to 7.0 at room temperature" (10). (b) " . . . above pH 7.0 the toxin was rapidly destroyed" (11). (c) "Rapid inactivation takes place in solutions above pH 7.0" (82). (d) During purification of the toxin the pH was not allowed to go above 6.5 (9). (e) The highest pH attained during purification of the toxin by Duff et al. (68) was 6.8. (f) Chromatography of the crystalline toxin on DEAE-cellulose was attempted at pH 6.5, and its fluorescence was studied at pH 6.8 (83). (g) Standardization of the crystalline toxin's mouse lethality was done in solutions of pH 6.2 (84). (h) The toxicity of a batch of crystallized type A toxin was found stable at pH 8.0 and 9.0 at 5°C for over 2 months (85). The method used to isolate the toxin appears different from that of Duff et al. (68), but the exact details were not given (85).

Williams et al. (81) have found that the homogeneous preparation of type A NT, stored at 4°C in 0.15 M TRIS-HCl buffer, pH 7.9, is stable for several months. The pure preparations of NT types A and E at very low concentrations (such as 1×10^{-10} M and lower) in physiological buffers, with and without added gelatin or serum albumin, are highly active (25,48,59,73,86). The effect of long-term storage on the pure NTs at high dilutions is not apparent in the published literature. The NT has constant physicochemical composition. Amino acid analysis of multiple batches of type A NT gave reproducible composition (87), and the same is true for type B and type E NTs (see refs. in 88). The amino acid compositions of the L and H chains after separation also show constancy; the sum of the amino acid contents of the two subunits equals the amino acid content of the NT (88). Table 3 shows (1) that the total number of the amino acid residues of the L and H chain of type A, within experimental error, is equal to that of the NT, and (2) that the amino acid composition determined empirically from acid hydrolysis of the protein matches, within experimental error, the composition derived from the amino acid sequence of the NT predicted from nucleotide sequence. Furthermore, those segments of the

Table 3 Number of Amino Acid Residues in Type A Neurotoxin (NT) and the Separated Light (L) and Heavy (H) Chains Determined by Amino Acid Analysis, and Comparison with the Amino Acid Residues of the NT Predicted from Nucleic Acid Sequence

| | From amino acid analysis (Ref. 88) | | | | From sequence (Ref. 31) |
|------------------|------------------------------------|-----|------------------|-----|-------------------------|
| | L | H | H + L | NT | NT ^a |
| Asp | 65 | 149 | 214 ^c | 200 | 78 |
| Asn ^b | — | — | — | — | 137 |
| Glu | 39 | 79 | 118 | 114 | 76 |
| Gln ^b | — | — | — | — | 39 |
| Ser | 28 | 55 | 83 | 79 | 84 |
| Gly | 30 | 37 | 67 | 64 | 64 |
| His | 6 | 6 | 12 ^d | 14 | 13 |
| Arg | 15 | 30 | 45 | 43 | 43 |
| Thr | 33 | 39 | 72 | 75 | 71 |
| Ala | 20 | 35 | 55 | 53 | 54 |
| Pro | 20 | 21 | 41 ^c | 44 | 38 |
| Tyr | 25 | 49 | 74 | 71 | 74 |
| Val | 26 | 40 | 66 ^c | 70 | 72 |
| Met | 7 | 17 | 24 ^c | 22 | 23 |
| Ile | 30 | 82 | 112 | 111 | 119 |
| Leu | 40 | 76 | 116 ^d | 104 | 113 |
| Phe | 34 | 37 | 71 | 68 | 70 |
| Lys | 39 | 63 | 102 | 100 | 103 |
| Cys | 4 | 6 | 10 | 10 | 9 |
| Trp | 4 | 16 | 20 ^d | 17 | 15 |

^aThe NT sequence, predicted from the nucleotide sequence, gives the amino acid composition of the single-chain protein before nicking. After nicking, 10 residues are excised (Kriegstein, DasGupta, and Henschen, to be published); subtraction of Thr-Lys-Ser-Leu-Asp-Lys-Gly-Tyr-Asn-Lys from this column makes agreement between this and the preceding column closer.

^bAsn and Gln were determined as Asp and Glu after acid hydrolysis.

^cDeviations between the sum of amino acid residues of H and L, and the parent NT: >5 and <10%; ^d>10%; all others <5%.

NT that have been analyzed for amino acid sequence (based on direct protein sequencing) match with the sequence predicted from the nucleotide sequence (not shown in the table). Also, the sum of the contents of secondary structure elements (α -helix, β -sheet, etc.) of the separated L and H chains equals the content of such elements of the parent molecule; thus the structural domains of the L and H chains are stable.

Toxicity Assays

In a very well conceived plan, the mouse lethality assay of the crystallized type A toxin was rigorously evaluated (84). Using one standardized toxin preparation, assays carried out in 11 independent laboratories according to a single prescribed protocol gave an average value of 0.043 ng toxin equivalent to 1 mouse LD₅₀ (the highest and lowest values were 0.075 and 0.032 ng, respectively; standard deviation was 0.012). Thus, 1 ng toxin is equivalent to 23.2 LD₅₀. This has been regarded as the recommended standardized potency of the toxin preparation and assay procedure (19), yet this same publication (19) defined 1 ng as equaling 30 mouse LD₅₀ without accounting for the difference between the values of 23.2 and 30 LD₅₀ per ng toxin.

The range in mouse lethality results noted in the 11-laboratory study was thought to be due to variation in the response of mice to toxin in each laboratory (84). In this context, consideration of the importance of correct placement of the inoculum during intraperitoneal injection appears relevant. Studies with substances other than botulinum toxin have revealed a 14% (89), 10–20% (90), and 12% (91) error in the placement of intraperitoneal injection of mice with a one-person procedure of injection. All or part of the misplaced inoculum was injected into the lumen of the stomach, the small bowel, or the uterine horn, or was injected subcutaneously, retroperitoneally, or intravascularly. The incidence of error was consistently reduced to 1.2% with a two-person procedure of injection (91).

The assertion that "the only means of evaluating the potency of acetylcholine-blocking power of the toxin is an animal assay [and that] there is no known . . . biological or immunological test available that can replace the mouse test for toxicity evaluation" (19) seems to preclude exploitation of an important alternative approach that actually monitors muscle paralysis, i.e., assessing the immediate postsynaptic effect of poisoning within 3 hr rather than recording the number of mice dying up to 72 hr.

Nerve-muscle preparations used for electrophysiological studies of neuromuscular junctions are useful for assaying the paralyzing effects of botulinum NT. Besides the classic phrenic nerve-hemidiaphragm (14), the plantar nerves-lumbrical muscles of the hind paw of the mouse (86) and the chick ciliary ganglion-iris muscle preparation (59) have been tested for their response to type A and E NTs. Some of these data, including the comparative response of phrenic nerve-hemidiaphragm preparations from various animal species to type E NT, are summarized in Table 4. In these nerve-muscle preparations the relationship between paralysis time (the time elapsed from addition of NT to tissue bath to loss of neurogenic response) and NT concentration (within a certain range) was linear or approximately linear. For example, when the crystalline type A toxin and rat phrenic nerve-hemidiaphragm were used, a plot on logarithmic coordinates of toxin concentration (3×10^{-9} to 1×10^{-11} M) versus paralysis time (80–300 min) yielded a straight line (14). Paralysis time (36–145 min) of mouse plantar nerve-lumbrical muscle was approximately linearly dependent on the concentration range of pure type A NT (1×10^{-9} to 1×10^{-11} M). The chick ciliary ganglion-iris preparation also exhibited muscle paralysis

Table 4 Paralysis of Nerve-Muscle Preparations by Various Concentrations of Type A and E Neurotoxins

| Toxin | Toxin Concentration (M) | Paralysis time (min) | Species and tissue | Reference |
|-------------------------------------|---|---------------------------|--|-----------|
| Crystalline type A toxin | 3×10^{-9} – 1×10^{-11} | 80–300 ^a | Rat phrenic nerve-hemidiaphragm | (14) |
| Pure type A neurotoxin | 1×10^{-9} – 1×10^{-11} | 36–145 ^b | Mouse plantar nerve-lumbrical muscle | (85) |
| Pure type A neurotoxin | 1×10^{-7} – 1×10^{-9} | 24–80 ^c | Chick-iris | (59) |
| Pure type E neurotoxin ^d | 3.4×10^{-10} – 3.4×10^{-11} | 44–89 ^b | Mouse plantar nerve-lumbrical muscle | (85) |
| Pure type E neurotoxin ^d | 1×10^{-11} – 1×10^{-13} | 80–320 ^c | Mouse phrenic nerve-hemidiaphragm | (25) |
| Pure type E neurotoxin ^d | 1×10^{-11} | 75 \pm 7 ^c | Mouse phrenic nerve-hemidiaphragm | (25) |
| Pure type E neurotoxin ^d | 1×10^{-11} | 104 \pm 10 ^c | Rat phrenic nerve-hemidiaphragm | (25) |
| Pure type E neurotoxin ^d | 1×10^{-11} | 97 \pm 10 ^c | Hamster phrenic nerve-hemidiaphragm | (25) |
| Pure type E neurotoxin ^d | 1×10^{-11} | 101 \pm 8 ^c | Guinea pig phrenic nerve-hemidiaphragm | (25) |

^aTime to reach 90% paralysis.

^bTime to reach complete paralysis.

^cTime to reach 50% paralysis.

^dIn all cases type E is dichain (after trypsinization).

in a dose- and time-dependent fashion within a range of 1–100 nM type A NT and 0.5–100 nM type E NT concentrations.

Looking Ahead

A recent opinion (19) considering the production and purification of botulinum toxins for clinical use, in accordance with appropriate standards of quality, safety, and good manufacturing practice, is notable: "These restrictions required culturing in simplified medium without the use of animal meat products and purification by procedures not involving synthetic solvents or resins [and] avoided exposures to substances such as added enzymes or columns of synthetic resins, used in some methods, that could contaminate the preparation and be carried into the final injected preparations . . . We do not recommend the use of methods of purification involving enzymes, various exchangers, or synthetic solvents because of the chance of contamination."

This view, on the side of caution (for safety), appears to contain the following contradictions. Toxin production (68) starts with a medium containing animal meat products—beef infusion and chopped meat; before crystallization, the toxin is precipitated with ethyl alcohol, a synthetic solvent. The toxin is filtered for sterility and stored in the presence of human serum albumin (19). Chromatography at present is a highly dependable and reliable technique that yields pure products with extraordinary reproducibility. If one assumes that column resins may somehow be a possible source of contamination, the same degree of possibility should then be applicable to the filters used for sterile filtering the toxin because "something" may leach out of the filter material.

The recommended extremely conservative guidelines only perpetuate the technology of 1946 (10), slightly modified in 1957 (68), and merely discourage the use of improved-quality crystallized type A toxin (72) and investigation of the clinical use of the pure NTs (mol. wt. 150,000) and chimeric toxins, because their production will require chromatography. The recommendations from the investigators in England (92), are prudent and more forward-looking. Interestingly, the quality of the toxin prepared in England with modern protein chemistry techniques does not rely on its optical properties (see ref. 48 and 11 in 92), presumably because of the fallacies discussed earlier.

Secure information on the structure of the NTs (mol. wt. ~150,000), the biological activities of the different segments of the NT molecule, the structure-function relationship, and the mechanism of action of the NT is rapidly emerging. It is hoped that more imaginative and courageous scientists and clinicians will team up to further exploit the new information to provide supportive insight into the clinical application of the NT and refinements in the regimen and response.

NOTE ADDED IN PROOF

The neuronal proteins (and some of their cleavage sites) proteolytically cleaved by botulinum neurotoxin serotypes A, C, D, E, and F, which were identified after this chapter was written, are as follows: neurotoxin serotypes A and E cut SNAP25 (soluble NSF attachment protein of mol. wt. 25,000), serotype C cuts syntaxin, serotypes D and F cut VAMP isoform 2. Unlike serotype B, which cuts VAMP between Gln 76-Phe 77, type D cuts VAMP between Ala 67-Asp 68 as well as between Lys 59-Leu 60, and type F cuts VAMP between Gln 58-Lys 59. In contrast to VAMP (vesicle-associated membrane protein), syntaxin is a protein embedded on the acceptor membrane and SNAP is cytoplasmic protein that associates transiently with membranes (93-95). A diagram on p. 488 of Ref. 95 illustrates the relative positions of these proteins involved in docking of the vesicle with presynaptic membrane.

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THOUGHTS ON ACTION OF BOTULINUM TOXIN
SUGGESTED BY REVERSIBILITY OF HEART EFFECTS

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Botulinum neurotoxin poisoning prevents the release of the neurotransmitter acetylcholine into the synaptic cleft. Stimulation of the cholinergic nerve is required for the acetylcholine-containing vesicles to attach to the inner surface at specific points of the nerve cell synaptic membrane. Thereupon at these points of attachment the acetylcholine leaves the vesicles to escape into the synaptic cleft by passage across the synaptic membrane. This event has been called exocytosis and is probably the action frustrated by the presence of toxin.

Classically botulinum neurotoxic activity has been considered to be persistent and almost irreversible. Recently cardiac effects of the toxin have been observed to be readily reversible and thus not persistent.^{2,3} I suggest that this paradoxical cardiotoxicity does not challenge the theory of inhibition of cholinergic nerves and that in fact the cardiac phenomena can contribute to productive thinking about the mode of action of botulinum toxin.

Type A hemagglutinin-free toxin causes electrocardiographic changes and a decreased heart rate (bradycardia) within minutes of injection of the toxin in rodents and dogs, the species studied. Within a short time there follows a spontaneous recovery to the normal heart picture. While this spontaneous recovery is not seen *in vitro* with isolated heart preparation a rapid reversal of cardiac effects does follow simple isotonic washing out of the toxin-containing fluid bathing the isolated heart. Another significant finding is repetitious bradycardia following each of successive exposures to toxin of the isolated heart preparation after washing out of the toxin.

The persistence of bradycardia with *in vitro* isolated heart preparations is due to the continuing presence of toxin in the fluid bathing the heart. *In vivo*, after injection of toxin there is a drop in blood level as the toxin is distributed and bound to nerve cells. The heart with its apparent weaker strength of toxin binding loses the toxin to places of greater binding capacity.

Repeated *in vivo* injections of toxin result in repetition of the bradycardia-spontaneous recovery cycle. It can be adduced that spontaneous recovery by *in vivo* exposure to toxin does not mean there is any fundamental difference in nature between *in vivo* heart and *in vitro* isolated heart toxicities.

Ready reversibility of cardiac toxicity and an unimpaired capacity for toxin uptake after prior uptake and washing out of toxin suggest that the intermolecular forces binding toxin to susceptible nerve cells do not lead to profound change in the cellular physicochemical environment for nerve reception of the toxin. This is more consistent with an adsorption type

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Effects. Lamanna; p 333-343.

of attraction than chemical bonding, and is not indicative of an enzyme action. With enzymatic activity an exhaustion of substrate would be expected.

The exposure to toxin does not cause any discerned permanent change in the nature of the toxin and membrane receptor. Concomitantly the toxin must be bound to the synaptic membrane for the entire period of poisoning, which can be days, weeks and months long for both experimental and natural botulinum poisoning.

Two possibilities are reasonable to consider for the long-term persistence of nerve cell dysfunction. The cytochemical lesion of botulism is so profound as to resist rapid repair. The alternative I favor is that paralysis persists for as long a time as the toxin remains lodged at the synaptic membrane. The half-life of toxin in the membrane can be a long one since there are no clues to suggest rapid catabolic destruction of the embedded toxin as, for example, by proteolysis. Cytological study of repair of poisoned nerve fibrils is consistent with persistence of membrane-associated toxin. Repair is slow in becoming visible, and significantly the growth of new functional fibrils in the poisoned nerve cell takes place outside the area of the preexisting poisoned fibrils.¹ Studies should be done for detecting continuous presence of toxin in the poisoned fibrils. This would provide definitive knowledge of the time scale of residence of toxin in the synaptic membrane and suggest if the toxin's presence hinders self-repair of the membrane site toxin occupies.

The following scenario is presented for botulinum neurotoxicity which I believe is consistent with the findings of cardiac studies.

Nerve stimulation is accompanied by two events: attachment of acetylcholine-containing vesicles to specific areas of the interior surface of the membrane and exocytosis of the vesicle. With toxin present nerve stimulation permits membrane uptake of the toxin and vesicle attachment to the synaptic membrane but exocytosis does not take place. Thus poisoned nerve cells do not accumulate acetylcholine in the cytosol. Such intracellular increase of free acetylcholine should happen if acetylcholine was released from vesicles and the released acetylcholine was not able to move across the nerve cell membrane into the synaptic cleft. Toxicity is explained if exocytosis were recognized to be a synaptic membrane-vesicle membrane interaction frustrated by toxin being present at this membrane-to-membrane interface.

Nerve stimulation acts as a valve which opens the nerve membrane to uptake of toxin. It is also accompanied by much studied intracellular and synaptic membrane changes in ionic environment in which calcium is a major participant. Conceivably toxin in the synaptic membrane disturbs access of ions to the trigger spot for exocytosis at the synaptic membrane-vesicle membrane interface. Exocytosis could be prevented by the toxin physically masking the trigger spot. This prevents contact of the proper ions with the trigger spot. Removal of the toxin which yields reversible cardiac toxicity means that the toxin resides in the synaptic membrane causes no irreversible changes in the events associated with initiation of exocytosis and movement of acetylcholine across the synaptic membrane. This is consistent with rapid recovery following removal of the toxin.

An explanation of botulism poisoning must account for differences in sensitivity to toxin among the sympathetic, parasympathetic and central nervous systems in spite of some common possession of cholinergic neurons. There might be differences among these systems in barriers affecting diffusion of toxin from lymph into the synaptic cleft. There may be a limiting width of the synaptic cleft that must be exceeded to allow entrance of the large-sized 150,000 dalton molecular weight toxin, a factor which might operate in the densely packaged brain. The cardiac effects at this stage of knowledge tell us nothing about the possibilities.

A prime factor affecting toxicity would be varying strength of binding forces of synaptic membranes holding toxin. These binding forces could differ among cholinergic neurons associated with different parts of body tissues and organs. If there is a spectrum from high- to low-strength binding forces the heart would appear to represent a marginal- or low-strength binding entity. A task that needs to be undertaken is to identify the sites and nature of toxin-

susceptible cardiac innervation. What can be said is that the toxin does not affect the intrinsic automaticity of the beat of isolated heart cells. It can be said that toxin inhibition of heart rates rests on nervous system control at sites located in the heart.

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Cardiac Effects of Botulinal Toxin

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Abstract—Crystalline type A botulinal toxin rapidly caused temporary bradycardia and electrocardiographic (ECG) changes in mice, rats, rabbits and dogs. In addition, in the dog the force of contraction was measured and found to be depressed. The ECG changes were indicative of conduction defects. The hemagglutinin present in the toxin played no role in the effects on the heart, since a derivative toxin without hemagglutinin also caused these phenomena. The cardiac effects were spontaneously reversible in the intact animal without removal of the toxin. On the other hand, in the *in vitro* isolated heart of the rat, recovery from the cardiac effects occurred only after the toxin was washed out of the preparation. The findings are consistent with, but do not prove, a physical rather than a chemical mechanism for the effects of toxin on the heart.

Introduction

Little attention has been paid to the occurrence of cardiac effects caused by botulinal toxin. Recent reviews of botulism poisoning (Simpson, 1981; Thesleff and Lundh, 1979; Thesleff, 1981) have barely mentioned cardiac levels or do not discuss the subject at all. In clinical studies (Petty, 1965; Ciccarelli and Gimenez, 1981; Sonnabend and Sonnabend, 1981), disorders of the heart either were not reported or were not considered to be characteristic responses in human poisonings. Effects of botulinal toxin on the isolated cat heart (Rosenblum, 1966) and on the vagus innervation of the heart of the cat, dog, rabbit (Dickson and Shevsky, 1923) and frog (Erzina and Mikhailov, 1956) have been reported. In this past work, no account was taken of the existence of hemagglutinin associated with the neurotoxin

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(Lamanna and Lowenthal, 1951). Thus the possibility has not been ruled out that the effects observed were due to extraneous material in the toxic culture fluid or to the hemagglutinin associated with the neurotoxin. Here we describe the cardiac effects of progenitor crystalline type A botulin toxin containing hemagglutinin and of the hemagglutinin-free derivative neurotoxin on the mouse, rat, rabbit, and dog as observed in electrocardiographic (ECG) recordings. Described is a previously unreported spontaneous and rapidly reversible bradycardia caused by the toxin.

Methods

All observations were made on the spontaneously beating heart without the use of experimental stimulation. This differs from past work cited, in which experimental stimulation of heart preparations was employed.

Toxin

Crystalline type A toxin that had hemagglutinin as a constituent was provided by E. J. Schantz of the University of Wisconsin. G. Sakaguchi, University of Osaka Prefecture, provided a purified sample of type A toxin free of hemagglutinin (Sakaguchi *et al.*, 1981). The toxins were dissolved in a sterilized phosphate-0.2 % gelatin buffer (pH 6.2-6.7) for storage and i.v. injection; this diluent in the amounts used and without toxin did not cause any discernible physiological changes in laboratory animals. Dosages of the toxin were measured as mouse LD₅₀ units.

Type A antitoxin and toxoid

These reagents were made available by the Centers for Disease Control of the U.S. Public Health Service, Atlanta, Ga.

Experimental animals

Male and female animals were used. The mice were Charles River CD1 and ICR strains weighing 25-35 g, and the rats were Charles River Sprague-Dawley and Wistar strains weighing 300-500 g. The New Zealand white rabbits weighed 3-4.5 kg and the dogs, adult beagles from the Food and Drug Administration-bred colony, weighed 9-12 kg.

Physiological measurements

A Hewlett-Packard polygraph was used to record measurements of physiological responses before and after exposure to toxin. Heart rates and

ECGs were recorded under light anesthesia induced by i.p. injection of sodium pentobarbital (mouse 15 mg/kg, rat 25 mg/kg, rabbit 10 mg/kg). A few unanesthetized mice and rats were also studied and showed no difference in measurements compared with anesthetized animals. Subcutaneous electrodes were inserted in appropriate limbs for monitoring the Lead II ECG and heart rate. After readings stabilized the toxin was given i.v.

In the dog, respiration, heart rate, ECG recordings and arterial blood pressure were monitored. The polygraph used was other than the one for the smaller laboratory animals. The dogs were anesthetized with sodium pentobarbital (35 mg/kg, i.v.). Cannulas were inserted in the right femoral vein and artery for drug injection and blood pressure measurements, respectively. Electrodes were inserted in the s.c. tissue of appropriate limbs for monitoring the Lead II ECG and heart rate. Respiration was recorded from a cuffed endotracheal tube inserted in the trachea and connected to a differential pressure transducer.

Isolated heart preparation

The spontaneous heart rate was measured for rats and dogs. Vagal nerves were not preserved and no experimental stimulus, either electrical or chemical for the beat was provided for the isolated heart preparations.

For rats, a Langendorff preparation was used with 52 cm of water perfusion pressure. The beating heart was rapidly removed from the body of an anesthetized rat and placed in pH 7.2 isotonic Krebs-Henseleit solution. The aorta was tied to a plastic cannula for continuous perfusion with Krebs-Henseleit solution kept at 36° C. The solution was oxygenated with a mixture of 95 % O₂ and 5 % CO₂. The perfusate from the heart was allowed to drip continuously and was collected in a graduated cylinder for measuring flow rate. The heart rate was recorded by means of a pair of electrodes attached to the surface of the heart and connected to a Hewlett-Packard polygraph for ECG recording. The preparation was allowed to stabilize for 5 to 15 min before the toxin solution was injected into the aortic cannula in a volume of 0.2 to 0.5 ml. After exposure to the toxin, the preparation was washed out with fresh toxin-free Krebs-Henseleit solution and allowed to reequilibrate before a second exposure to toxin.

The isolated dog heart preparation has been described by Vick and Herman (1971). The heart was excised from an anesthetized dog and perfused with 500 ml of heparinized autologous blood. Heart rate, perfusion pressure, ECG and force of contraction were measured continuously. The toxin was injected directly into the inflow side of the perfusion tubing and allowed to recirculate throughout the experiment.

Results

The crystalline toxin and the hemagglutinin-free toxin had the same qualitative effects on the heart, namely bradycardia and changes in the ECG. Thus, hemagglutinin apparently does not play a role in the cardiovascular changes resulting from exposure to the toxin. On a weight to weight basis, hemagglutinin-containing toxin was less potent than the hemagglutinin-free toxin in causing cardiac changes; a larger amount was needed to produce the effects and recovery was more rapid (Tables I and II, compare where toxin doses overlap). This greater potency might be thought to be related to the smaller molecular size of the hemagglutinin-free toxin (Lamanna *et al.*, 1970). On the other hand, potency expressed as molar specific activity (number of molecules required to cause a given effect) does not indicate the crystalline toxin to be less active in causing cardiac effects than the hemagglutinin-free toxin. The crystalline toxin has a molecular weight of 900,000 and possesses 240×10^4 mouse LD_{50} /mg nitrogen while the hemagglutinin-free toxin has a molecular weight of 150,000 and possesses 500×10^4 mouse LD_{50} /mg nitrogen (Sakaguchi *et al.*, 1981). Thus the weight of crystalline toxin needed for a particular biological activity would have to be at least 3 times greater than for a hemagglutinin-free toxin before it could be said to be less potent in terms of molar specific activity. There is still another way of thinking of potency. If the assumption is made that the same number of cell receptors are tied up for the same LD_{50} of the large and small molecule toxins, then the finding (Lamanna *et al.*, 1970) that the toxic effects of the small molecule are elicited more rapidly than those of the large molecule, permits saying that the small molecule is more potent. The definition of potency elected affects the judgment of relative potency of the different molecular size toxins. It is also a fact that crystalline toxin can dissociate with separation of the hemagglutinin component from the neurotoxic component. Such a separation has been reported in the case of i.v. injection of rabbits with crystalline toxin (Hildebrand *et al.*, 1961). The extent of disassociation varies with solvent conditions so that the situation for crystalline toxin dissolved in blood and blood-free salt solutions cannot be considered to be the same. We do not know the extent and variability in separation of the hemagglutinin from the crystalline toxin under our experimental conditions, so definite conclusions about relative potency of the 2 states of the toxin are not possible.

In mice and rats individual responses varied greatly. To date dose-response curves have not been obtained because of the large number of animals that would be required. However, the data show a trend for prolongation of lag time and a quicker recovery from bradycardia with decreases in toxin doses. At doses that caused death in a few hr, complete

TABLE I
Heart rates (% of normal rate) of individual mice after i.v. injection of hemagglutinin-free botulinal toxin (¹)

| Time after toxin injection (min) | Toxin dosage (mouse LD ₅₀ units) | | | | | | | | | |
|--|---|-----------|----------|---------|---------|----------|---------|--------------------------|--------------------------|--|
| | 82,000 | 20,500 | 5,000 | 1,025 | 300 | 38 | 19 | 5 | 3 | |
| 5 | 78, 80 | 91, 90 | 90, 89 | 94, 100 | 63, 83 | 90, 94 | 94, 90 | 100, 105 | 92, 83 | |
| 15 | 70, 67 | 70, 61 | 58, 60 | 88, 100 | 53, 74 | 55, 60 | 59, 71 | 83, 95 | 100, 74 | |
| 30 | 45, 44 | 56, 50 | 74, 50 | 72, 72 | 58, 58 | 44, 60 | 47, 100 | 83, 81 | 110, 95 | |
| 45 | 40, 40 | 56, 90 | 105, 90 | 59, 40 | 84, 48 | 40, 60 | 47 | 100, 71 | | |
| 60 | 35, 44 | 56 | | | 106, 81 | 44, 76 | 59 | 100, 92 | | |
| 75 | 30, 40 | 56 | | | | 106, 106 | 71 | | | |
| 90 | 35, 55 | 62 | | | | | 82 | | | |
| 105 | 56, 72 | 70 | | | | | | | | |
| Approximate time of death (hr) | 5, 3.33 | 4.25, 2.5 | 2.8, 2.8 | 24 | 24 | 24 | 24 | Alive after 5 days | Alive after 5 days | |

(¹) Heart rate at 0 time was considered as 100 %.

TABLE II

Heart rates (% of normal rate) of individual mice injected i.s. with crystalline botulinum toxin containing hemagglutinin (¹)

| Time after toxin injection (min) | Toxin dosage (mouse LD ₅₀ units) | | | | |
|--|---|--------|----------|---------|----------|
| | 4000 | 400 | 40 | 20 | 10 |
| 5 | 80, 83 | 95, 86 | 100, 100 | 94, 91 | 100, 96 |
| 15 | 70, 72 | 83, 61 | 74, 100 | 77, 70 | 105, 80 |
| 30 | 70, 89 | 90, 61 | 70, 97 | 124, 91 | 100, 120 |
| 45 | 75, 117 | 90, 86 | 90, 113 | | 105, |
| 60 | 85 | 103 | | | |

(¹) Heart rate at 0 time was considered as 100 %. All mice died within 12 hr of exposure to toxin.

TABLE III

Effect of botulinum toxin on ECGs

| Species | P-R interval | QRS | Q-T | Type of arrhythmia |
|--------------------|-----------------|-----------|---------------------------|---|
| Mouse | | | | |
| Intact | Prolonged (*) | Prolonged | Prolonged | Bradycardia |
| Rat | | | | |
| Intact | Prolonged | Prolonged | Prolonged | Bradycardia |
| Isolated heart (*) | — (*) | — | — | Bradycardia and AV premature beat |
| Rabbit | | | | |
| Intact | Prolonged | Prolonged | Prolonged | Bradycardia and bigeminal rhythm |
| Dog | | | | |
| Intact (*) | — | — | S-T segment depression | Bradycardia, bigeminal rhythm, and ventricular extra systole |
| Isolated heart | — | — | — | Bradycardia and ventricular fibrillation |

(*) Prolonged means extended in time.

(*) Flow rate decreased in isolated rat heart.

(*) Dash indicates not measured.

(*) The data recorded are effects unaccompanied by respiratory difficulties of the animal. Variable blood pressure changes, decreased contractility, and increased perfusion pressure were observed in the isolated dog heart.

TABLE IV

Heart rate (% of normal rate) of the isolated rat heart with repeated exposure to botulinal toxin (¹)

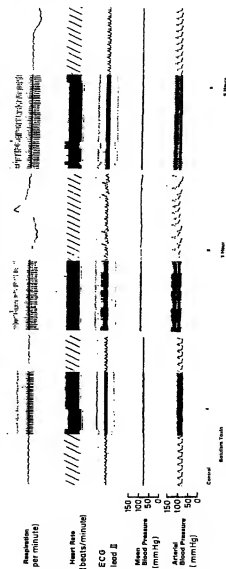
| Time of successive exposure (min) | Recovery | Lowest value |
|--|----------|--------------|
| Hemagglutinin-free toxin (41,000 mouse LD ₅₀ units) | | |
| 0 | 100 | |
| 0-10 | 90 | 72 |
| 10-20 | 96 | 72 |
| 20-30 | 96 | 66 |
| 30-70 | 96 | 78 |
| 70-100 | 84 | 66 |
| 100+ | 90 | 72 |
| Hemagglutinin-containing toxin (20,000 mouse LD ₅₀ units) | | |
| 0 | 100 | |
| 0-5 | 108 | 66 |
| 5-10 | 78 | 66 |
| 10-15 | 90 | 72 |
| 15-20 | 90 | 72 |
| 20+ | 84 | 60 |

(¹) Toxin was flushed out of the preparation within 2 min after exposure and the heart rate recovered within 10 min of the exposure. Toxin was reintroduced into the preparation at 10-min intervals.

recovery of the normal heart rate before death was often incomplete (Table I). At low doses that killed rats and mice many hr or days after injection, full cardiac recovery was reached before the animal died. Reduction of the heart rate to below 30 % of the normal rate was not observed; in the majority of animals, the heart rate was in the range of 45-90 % of the normal rate and was only roughly dependent on the dose. The deaths of animals recorded in the Tables are attributed to respiratory paralysis because of signs of respiratory muscular failure. But these signs appear many minutes or hours after the more rapidly occurring reductions in heart beats. Thus the effects of the toxin on the heart and respiratory paralysis are independent events.

Thesleff and Lundh (1979) and Thesleff (1981) showed that above a certain quantity of toxin there is no further effect on release of neurotransmitter. In other words, when the cellular receptors for the toxin have been saturated at a particular dose, increases beyond this dose are without further effect. Consistent with this is the lack of reduction in heart rate below about 30 % of normal. This probably represents the point of

Effect of Botulism Toxin on Heart Rate, ECG, Respiration and Blood Pressure in the Dog



saturation of cellular receptors by the toxin and the residual automaticity in the heart rate not subject to poisoning by the toxin.

Table III summarizes representative ECG findings for the rat and rabbit. The cardiac effects were preventable by use of specific antitoxin at doses preventing death. Toxoid was without effect when given to the rat at a dose of 0.014 mg nitrogen which is equivalent to 304×10^6 mouse LD₅₀ of crystalline toxin based on a reported value of 240×10^6 mouse LD₅₀/mg nitrogen of crystalline toxin (Sakaguchi *et al.*, 1981). Slowing of the heart rate was the prominent common feature of exposure to toxin. This bradycardia had the following characteristics: in the intact rat and rabbit, 5–30 min elapsed before slowing of the heart rate occurred. The effect in the intact animal was spontaneously reversible and the disturbance could last only a short time (30–60 min); a single lethal dose could cause the cardiac effects, with death of the toxin-poisoned animals occurring hours or days after recovery of the heart rate.

In control rats injected with anesthetic and toxin-free diluent the heart rate over a 2-hr period varied over a range of 93–114 % of normal. Increased heart rate was not observed in the toxin injected rats. The heart rate for 13 anesthetized rats was 430 beats/min \pm 5 standard error of the mean while the rate for 10 isolated hearts was 215 ± 11 .

Isolated rat hearts showed a rapid decrease in the intrinsic heart rate and the coronary flow rate decreased to 10–40 % of normal, with recovery taking place within min after toxin was flushed out (Table IV). Continuous perfusion with toxin in the perfusion fluid maintained the decrease in intrinsic heart rate during the entire perfusion period. Toxin neutralized with antitoxin did not affect the isolated rat heart preparation.

The administration of toxin to the various species produced reproducible ECG changes indicative of conduction defects. Thus, the Lead II ECG revealed prolongation of the P-R, QRS, and Q-T intervals (Table III).

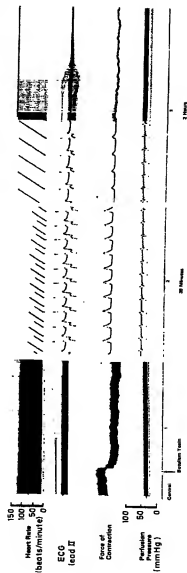
The dose of hemagglutinin-free toxin given to the dogs was about 4,000,000 mouse LD₅₀ units, which is about 4000 mouse LD₅₀ units per ml

FIG. 1

Effect of crystalline hemagglutinin-containing type A toxin (8×10^6 mouse LD₅₀ units) on respiration, heart rate, ECG, and blood pressure in the anesthetized adult beagle dog given toxin i.v. Note change in ECG and arterial pulse tracing 1 hr after injection of toxin and recovery at 5 hr. The toxin had no effect on arterial blood pressure or respiration during the first hr after injection; however, at time of arrhythmia, there was a bigeminal pulse trace.

Parameters measured are calibrated as follows: (1) Heart rate = beats per min, measured on a fast trace by counting each beat for 1 min; (2) ECG: intervals measured in msec, amplitude in mV; (3) Mean blood pressure = an electrical mean which averages systolic and diastolic pressure differences; (4) Arterial blood pressure = measured as systolic and diastolic pressure on a 0 to 200 mmHg scale; (5) Force of contraction = measured on a Walton-Brodie strain gauge.

Effect of Botulinum Toxin on Heart Rate, ECG, Force of Contraction and Perfusion Pressure in the Isolated Dog Heart



of blood, based on an estimated blood volume of 100 ml/kg in the dog. The dose led to death in 1 to 8 days after toxin exposure.

Nine anesthetized dogs were given the toxin i.v. The injection was followed by an immediate slight decrease in blood pressure and a variable increase in heart rate. This tachycardia was not seen in the other animal species and might be a temporary response to the stress of injection or an inhibiting effect on the vagal nerve. These changes returned to beginning values within 5 to 15 min after exposure to toxin. From 30 to 60 min, 6 of the 9 dogs began to show ECG changes; these consisted of depression of the T wave and, in 2 dogs, premature ventricular contractions (extrasystole) (Fig. 1). There were no changes in respiration during the time the ECG changes were occurring. The extrasystole was accompanied by a bigeminal pulse pattern that persisted until the ECG returned to normal (Fig. 1). Complete ECG recovery including bradycardia took place within 1.5 to 3 hr after exposure to toxin. The decrease in heart rate was in the range of 35 to 135 beats per min. The normal pulse in the anesthetized dog ranged from 180 to 210 beats per min.

Bilateral vagotomy, atropine at a level of 0.5 mg/kg and a combination of the two in dogs did not prevent arrhythmias, ECG changes, or bradycardia from occurring after the toxin injection. The results were the same when atropinized (1 mg/kg, i.p.) rats were given the toxin. At the peak of the ECG effects 2 dogs were given antitoxin sufficient to neutralize the toxin given. No alteration in cardiac response was observed except that one of these dogs survived the exposure to toxin.

The isolated perfused dog heart responded to toxin (8000 mouse LD₅₀/ml in 500 ml of perfusate) in much the same manner as did the intact animal. The heart rate of the isolated dog heart was 90–105 beats/min and for the dose of toxin used dropped to 75–90 beats/min. These changes would not be expected in a control unchallenged heart preparation (Vick and Herman, 1971). There was a persistent decrease in both the heart rate

FIG. 2

Effect of crystalline hemagglutinin-containing type A toxin on heart rate, ECG, force of contraction and perfusion pressure of an isolated dog heart perfused with blood with toxin added. Note that at 30 min and 2 hr there was a progressive decrease in heart rate and force of contraction accompanied by a marked elevation in perfusion pressure. Following toxin, the heart rate decreased from an average of 90–105 beats/min to 75–90 beats/min (84.5 % of control). This heart failed completely at approximately 2 1/4 hr after having received the toxin.

Parameters measured are calibrated as follows: (1) Heart rate = beats per min, measured on a fast trace by counting each beat for 1 min; (2) ECG: intervals measured in msec, amplitude in mV; (3) Mean blood pressure = an electrical mean which averages systolic and diastolic pressure differences; (4) Arterial blood pressure = measured as systolic and diastolic pressure on a 0 to 200 mmHg scale; (5) Force of contraction = measured on a Walton-Brodie strain gauge.

and force of contraction throughout the 3- to 5-hr observation period (Fig. 2). In one heart there was an immediate ventricular fibrillation which was reversed by electrical defibrillation. The addition of antitoxin appeared to stabilize this heart preparation against further ventricular fibrillation. Of 4 isolated heart preparations, 2 failed at from 1 to 1.5 hr after toxin treatment with progressive loss of force of contraction and cardiac arrest.

Discussion

The cardiac effects of botulinal toxin are direct; they are not occurring as a response to respiratory distress or paralysis. This conclusion is based on a number of facts: (a) ECG effects can occur before signs of respiratory distress; (b) poisoned animals with obvious respiratory distress can have a normal ECG; (c) at autopsy immediately after death from respiratory paralysis, the heart may continue to beat and has a normal appearance; and (b) the strongest evidence of the independence of cardiac effects from respiratory distress is the occurrence of similar toxic effects on isolated *in vitro* heart preparations.

The reason that the cardiac effects have not been recorded in clinical studies of botulism, is probably that the victim does not seek medical attention early in the poisoning. Since the phenomenon is rapidly reversed, the clinician sees the patient at a time when the cardiac effects are no longer evident and support of respiration is the key to therapeutic success. Assuming the human heart is sensitive, ignorance of sensitivity relative to laboratory animals prevents any realistic guess as to the significance of the phenomenon in human botulism. Dr. V. R. Dowell of the Centers for Disease Control, USPHS, Atlanta, Georgia, tells us that in blood or serum from human victims of botulism rarely is there as many as 20 mouse LD₅₀/ml present with 1 or 2 LD₅₀ being more commonly detected.

The cardiac studies of Dickson and Shevly (1923) focused on the vagal nerve as the site of action of the toxin. However, vagus innervation may not be the only action of the toxin, since bradycardia was observed in isolated heart preparations. This finding is supported by our experience with vagotomy in the dog. In the intact animal, poisoning of the vagus nerve would predict the occurrence of tachycardia rather than bradycardia. Rosenblum (1966), in studies of the isolated cat heart, concluded that the toxin affected post-ganglionic parasympathetic nerves, but did not specify where in the heart these susceptible nerves were located.

A number of locations can be considered (Napolitano *et al.*, 1965; Löffelholz, 1981; Löffelholz *et al.*, 1982) as sites of action for the toxin: the junction of the vagal nerve with the sino-atrial (SA) node; site of SA-atrioventricular (AV) interaction; His bundle, Purkinje fibers and extranodal cardiac fibers. Our findings with the vagotomized and

atropinized animal and the isolated heart rule against the vagal nerve-SA junction as a primary site of action for the heart changes observed in our study.

Heart muscle cells isolated from the nervous system have an intrinsic capacity to beat. This beat did not change when toxin was added to rat ventricular muscle tissue culture, nor was the viability of the tissue culture cells affected. This finding is consistent with the lack of effect by the toxin in chick heart cell tissue cultures (Lamanna, unpublished observations, 1951).

A remarkable feature of the cardiac effects of the toxin is its ready reversibility. This is in contrast to the experience with other model nerve systems such as the commonly used phrenic nerve-diaphragm muscle *in vitro* preparation. Table IV illustrates this phenomenon of reversibility for both the hemagglutinin-containing toxin and the hemagglutinin-free toxin in the isolated rat heart. There was a striking difference between the intact and isolated heart in the reversibility of cardiac changes following exposure to toxin. In the whole animal, heart changes were reversible, even though exposure to toxin was continued. However, in the isolated rat heart, recovery occurred only after the toxin was washed out of the preparation. The *in vivo* heart, unlike the isolated heart, apparently possesses an adaptive means for reversing cardiotoxicity even with continued exposure to toxin (Tables II and V).

Reversibility of the bradycardia caused by botulinal toxin presumably did not occur in the isolated cat heart since it was not mentioned (Rosenblum, 1966). Vincenzi (1967) reported that, in the rabbit, the beat of the isolated SA-node upon stimulation was irreversibly and completely blocked by type E toxin culture supernatant. However, impure toxin in culture fluid was used in those studies and therefore the findings cannot be definitively compared with our results. In addition, the isolated hearts were not stimulated in our work, so the response measured was completely spontaneous.

Botulinal toxin is known to act on the cholinergic parasympathetic system (Thesleff, 1981). Actions of the toxin may be limited to inhibition of cholinergic nerves, including those in the heart. Because of the ready reversibility, the cardiac effects described in this paper may be a tool of unique value in studies of the sites and mechanisms of action of botulinal toxins. Further investigations of this heart poisoning may yield information about the nature of cardiac innervation that has not as yet been clearly recognized. Among the possibilities for future study is the determination of the absence or presence of linkages of parasympathetic synapses with adrenergic pathways in the heart (Löffelholz, 1981; Löffelholz *et al.*, 1982).

What is the significance of the reversibility of bradycardia and ECG changes for hypotheses of the mechanism of action of botulinal toxin? Reversibility favors, although it does not prove, a physical rather than a

chemical lesion at the site of poisoning by the toxin. For the case of a chemical cause of toxicity, the receptor or site of action of the toxin would be expected to undergo a change in structure with a concomitant loss in the capability to react anew with toxin molecules. Otherwise, for repeated and rapid uptake of toxin (Table IV) at a given specific receptor site, there must be an extraordinary rate of repair of chemical damages at sites of uptake or action of toxin. On the other hand, a physical mode of action, such as an adsorption-desorption phenomenon, would not require time-consuming and repeated in-place synthesis of receptor or other site of action damaged by toxin. Thus, the findings fit a physical mode of action, such as that in the "pipe and valve" hypothesis (Lamanna, 1976; Hanig and Lamanna, 1979; Simpson, 1981).

There may be 2 kinds of chemically different receptors, one in the heart which loosely binds toxin, and another elsewhere in the parasympathetic system which strongly binds toxin. Another possibility is the existence of a chemically identical receptor which differs in the site and manner of its placement in unlike tissues, with consequent variation in the accessibility or penetration of the toxin to the receptor. Either possibility can be accommodated by a purely physical mode of action.

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in partial fulfillment of the requirements for the
degree of Doctor of Philosophy

by

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**CHARACTERIZATION AND STABILIZATION OF
CLOSTRIDIUM BOTULINUM
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MICHAEL CHARLES GOODNOUGH

A thesis submitted in partial fulfillment of the
requirements for the degree of

**Doctor of Philosophy
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...and all this science I don't understand. It's just my job five days a week.

-Elton John, *Rocket Man*

CHARACTERIZATION AND STABILIZATION OF *CLOSTRIDIUM BOTULINUM* NEUROTOXIN USED MEDICALLY

Michael Charles Goodnough

Under the supervision of Associate Professor Eric A. Johnson,
at the University of Wisconsin-Madison

Clostridium botulinum produces one of the most potent neurotoxins known. This neurotoxin causes a flaccid paralysis by preventing the release of the neurotransmitter acetylcholine across the synaptic junction of the motor endplate. The neurotoxin is the causative agent of foodborne botulism, wound botulism, and infant botulism. In the past 10-15 years, the neurotoxin has been developed for use as a therapeutic agent in the treatment of focal dystonias and spastic muscle disorders by direct injection of nanogram quantities into overactive muscles. In this research, I have addressed issues related to the quality of botulinum toxins for medical use.

Differentiation of individual neurotoxin serotypes was accomplished by development of a sensitive colony immunoblotting assay that was capable of detecting toxin producing colonies as well as distinguish between high titer colonies and those producing less neurotoxin. Neurotoxin produced by colonies of *Clostridium botulinum* types A, B, E and toxigenic *Clostridium butyricum* bound to nitrocellulose was detected by an enzyme-linked immunoassay procedure. The procedure used serotype specific rabbit IgG as the primary antibody and goat antirabbit IgG antiserum labeled with alkaline phosphatase as the marker to visualize immobilized neurotoxin from individual colonies. The method differentiated the colonies based on serotype of neurotoxin produced and by the amount of neurotoxin produced by individual colonies on the same agar plate. Specificity of primary antibodies used was improved by adsorption of cross-reacting heterologous antibodies.

Neurotoxin associated with non-toxic proteins in high molecular weight complexes was characterized at each step in the purification process showing that the purification

process currently approved by the United States Food and Drug Administration is variable with regard to yield and specific toxicity. The toxin for medical use in the United States is produced by the Hall A strain as part of a complex of at least six other proteins and is purified by a series of precipitations and crystallizations. In this study, the toxin complex was examined at each step in the purification scheme for toxicity recovered, % solids, optical density at 260 and 278 nm, specific toxicity, and for the presence of ribonucleic acids. The results show that the procedure is variable and dependent to a large degree on the individual components making up the growth medium indicating nutritional regulation of toxin formation. Ribonucleic acid associated with the purified toxin complex was found at a level of 0.3% and did not appear to be specific.

Botulinal neurotoxin must be lyophilized or freeze-dried to allow for shipping and handling of the relatively delicate protein. Recovery of type A and B toxin activity following lyophilization was dependent on a number of variables. Conditions were found that gave >90% recovery of the toxicity following lyophilization of solutions containing 20-2,000 mouse 50% lethal doses. Full recovery of type A and B toxin complex toxicity as well as the purified ca. 150 kDa toxin molecule was obtained on drying 0.1 ml when the pH was maintained below 7.0 and serum albumins or other protein excipients were used as stabilizers in the absence of sodium chloride. Shelf stability was improved by addition of trehalose to the serum albumin system but not by addition of sucrose or maltotriose. This drying formulation allowed storage of lyophilized type A toxin complex and purified type A neurotoxin at temperatures up to 37°C for months with minimal losses in activity.

Inactivation events which occurred during drying of type A and B neurotoxin were investigated. The various drying processes and formulations cause varying degrees of inactivation of the toxin and formation of toxoid. This toxoid adds to the antigenic burden of the material and further increases the chances of patients developing neutralizing antibodies. The processes which result in the formation of this toxoid can involve

aggregation, deamidation, peptide bond hydrolysis, and oxidative degradation. In this study we demonstrated that in our model system using purified type A and B Clostridium botulinum neurotoxins, aggregation, deamidation, and peptide bond hydrolysis occur during the lyophilization procedure causing decreases in the specific toxicity. Among the events causing loss in specific activity of the neurotoxin, peptide bond hydrolysis at the aspartate 12-proline 13 residue in the light chain was shown in purified type A neurotoxin. Deamidation was shown to occur in both type A and B purified neurotoxins using radiolabel incorporation with protein isoaspartyl methyl transferase. Type B showed more deamidation than type A neurotoxin. Aggregation also caused losses of both neurotoxin serotypes during lyophilization.

Sub lethal doses of various preparations of Clostridium botulinum type A toxin including Botox®, and Dysport®, were tested in a rabbit model for immunogenicity. Known quantities of various type A toxin preparations were injected over a period of time and the animals serum assayed for antibodies capable of neutralizing a small challenge of purified type A toxin in mice. Animals injected with Botox® developed neutralizing antibodies to the toxin in 60 and 63 days. Animals injected with type A toxin complex produced in our laboratory produced antibodies that neutralized the challenge in 118 days while animals treated with purified type A neurotoxin and Dysport® did not produce neutralizing antibodies over the course of the 2-3 month injection schedule.

Approved E. F. [signature]

Date March 15, 1984

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Chapter I**Introduction**

Members of the genus Clostridium produce some of the most potent neurotoxins known. Within this genus, the species Clostridium tetani and Clostridium botulinum are probably the most widely known. Neuropoisonings due to these organisms have been recognized since antiquity. Hippocrates described a case of tetanus as follows: "The master of a large ship crushed the index finger of his right hand with the anchor. Seven days later a somewhat foul discharge appeared; then trouble with his tongue-he complained that he could not speak properly...his jaws became depressed together, his teeth were locked, then symptoms appeared in his neck; on the third day opisthotonus appeared with sweating. Six days after the diagnosis was made he died." Although caused by a similar neurotoxin, botulism is more subtle in its symptoms. In contrast to the spastic paralysis of tetanus, botulism causes a flaccid paralysis which may have gone undetected for many years as a disease. Niemann (1991) quotes an author of a textbook on poisons named Schanaq who described the production of a highly potent toxin: "Collect blood from the left vein of the neck of a black bull, fill it into an unrinsed sheep gut, seal it tightly and dry the content in the shade of a mulberry tree. The powdered residue intermingled with food will lead to death within three days." This author may have described the first commercial drying operation of botulinum toxin as well!

Botulinum toxin has been used therapeutically since the early 1980's in the treatment of spastic muscle disorders thanks in large part to the efforts of Drs. Edward J. Schantz and Alan B. Scott. My research into the nature and stabilization of botulinum neurotoxins is organized into eight chapters. The first is a brief, historical introduction. The second chapter is a review of the current literature. It is meant to give the reader a general background on the subject of Clostridium botulinum and its neurotoxin. It is by no means exhaustive. Further, more specific background information is presented in the introduction of the five succeeding chapters describing my research. These five chapters (III-VII) describe original research and are arranged in the format of research articles.

Material from chapters III and V was previously published in *Applied and Environmental Microbiology* while portions of chapter V have been accepted for publication by the American Chemical Society in a symposium series book. There is information in both chapters that has not been previously published.

Chapter III deals with detection and differentiation of botulinum neurotoxin in and around colonies grown on agar medium. Chapter IV describes the characterization of the type A botulinum toxin purification as done by Schantz for production of 79-11 and other lots of pharmaceutical grade toxin. Chapter V describes research on the stabilization of some of the various botulinum neurotoxins during lyophilization. Chapter VI deals with some of the possible mechanisms of inactivation that occur when toxin is lyophilized. Chapter VII describes an animal model for testing the immunogenic potential of different botulinum toxin preparations including two commercially available products. Finally, Chapter VIII contains conclusions and future prospects along with some commentary that did not seem to fit any other place in this thesis.

Reference:

- Niemann, H. 1991. Molecular biology of clostridial neurotoxins. In *Sourcebook of bacterial protein toxins*, J. Alouf and J. Freer (eds.). Academic Press Ltd., London.

CHAPTER II

Background and Significance

I. The organism.

Classification.

Bergey's Manual (Cato et al., 1986) classifies Clostridium botulinum as a gram positive, obligately anaerobic, endospore forming rod-shaped organism with general dimensions of ca. 1.5-2.0 x 3.0-6.0 μm . The organisms that are classified as C. botulinum have in common the characteristic ability to produce a very potent neurotoxin. This neurotoxin is proteinaceous in nature and acts on the pre-synaptic junction of motor-neurons and the muscle fibers innervated by the neuron. Botulinum neurotoxin is quantitated in terms of mouse intraperitoneal 50% lethal doses (LD₅₀), (Schantz and Kautter, 1978).

There are at present seven known serotypes of botulinum neurotoxin, A, B, C₁, D, E, F, and G. Two additional toxins are produced by some type C strains, C₂ and C₃ toxin, which are classified as cytotoxins due to their modes of action. These latter two clostridial toxins are ADP-ribosylating toxins as opposed to neurotoxins (Aktories et al., 1986; Moriishi et al., 1991). Generally, a given strain produces a single neurotoxin type but there are exceptions. Some strains have been known to produce two serotypes of toxin. One, type Af, produced 93% type A toxin and 7% type F toxin while another designated Bf produced 90% type B toxin and 10% type F (Hatheway, 1989). Types C and D strains are known for interconversion of toxin serotypes by phages (Smith and Sugiyama, 1988).

Other clostridia have been isolated that produce botulinum-like neurotoxins. An organism phenotypically resembling C. baratii was isolated in 1985 from a patient in New Mexico with infant botulism (Hall et al., 1985). This organism produced type F neurotoxin and was determined by DNA homology to be a toxigenic strain of C. baratii (Suen et al., 1988). Two separate cases of infant botulism in Italy were determined to have

been caused by an organism that phenotypically closely resembled *C. butyricum* but was capable of producing botulinum neurotoxin (McCroskey et al., 1986; Aureli et al., 1986). Again, DNA homology showed the organism to be *C. butyricum* (Suen et al., 1988).

C. botulinum strains are further classified phenotypically by proteolytic activity.

Group I organisms are those that digest coagulated egg white or meat particles and are considered to be proteolytic as well as saccharolytic; Group II organisms are those that are considered non-proteolytic and saccharolytic; Group III consists of those organisms that may or may not be proteolytic and produce one or more of the toxin serotypes mainly considered to affect birds and animals; while Group IV organisms are designated non-proteolytic and non-saccharolytic type G strains. A summary of the various serotypes and their culture group is shown in the following (adapted from Sugiyama and Sofos, 1988, and Rhodehamel et al., 1992):

| | |
|---|---------------------------------------|
| <u>Group I</u> (proteolytic saccharolytic strains) All type A strains, proteolytic type B and F strains. | <u>Sports</u> Oval and subterminal |
| <u>Group II</u> (non-proteolytic, saccharolytic strains) All type E strains, non-proteolytic type B and F strains. | Oval and eccentric to subterminal |
| <u>Group III</u> (proteolytic or non-proteolytic strains producing one or more of the following toxin serotypes) Type C ₁ , C ₂ , C ₃ , and D. | Oval and subterminal |

Group IV

(non-proteolytic, non-saccharolytic strains)

Oval and subterminal

All type G strains.

Group I and II strains are the predominant causes of human botulism while Group III strains are primarily known for causing avian botulism (Smith, 1982) as well as botulism of cattle, sheep (Smith, 1977) and some captive animals such as lions (Greenwood, 1985).

Growth requirements.

Most research attention has been focused on the growth of C. botulinum in foods for obvious reasons. The predominant factors involved are temperature, pH, water activity, redox potential, presence or absence of inhibitors and competing microflora. Most processed foods present a series of these "hurdles" acting together to prevent outgrowth of the organism and toxin formation.

Temperature requirements vary between the different groups. Group I organisms typically have a growth optimum of 37°C and a minimum of ca. 10°C with a maximum of 45-50°C. Group II organisms which are considered to be psychrotrophic and have growth optima of ca. 30°C; they are capable of growth and toxin formation at temperatures as low as 3.3°C. Maximum growth temperatures for group II organisms are in the range of 40-45°C (Hauschild, 1989).

Lower pH limits for growth of group I strains have been found to vary in the range of 4.6-5.0. For group II strains the limit is ca. 5.0. These limits are dependent on all other factors being close to optimum. The upper limit of growth for C. botulinum has been reported to be in the range of pH 8-9 (Hobbs, 1976).

As with all organisms, growth of C. botulinum depends on a minimum quantity of available water (Aw). Water activity in foods is usually controlled by the use of salt

(sodium chloride) or sugars. With other conditions such as pH, temperature, etc. being close to optimum, the maximum salt concentrations for growth of group I and II organisms is in the range of 10% ($A_w = 0.94$) for group I and 5% ($A_w = 0.97$) for group II (Genigeorgis, 1986).

Redox potential levels that will allow the initiation of growth of *C. botulinum* are quite high for an obligate anaerobe, being on the order of +200 mv if there are no other environmental stresses. One of the primary methods of inhibition of *C. botulinum* in cured meats is inclusion of nitrite and nitrate. Nitrate is reduced to nitrite which is the active compound. Nitrite is believed to react with iron-sulfur proteins such as ferredoxin to form iron-nitric oxide complexes. These claims are supported by the work of Tompkin et al. (1978). In their work, the addition of iron salts or iron ions counteracted the inhibitory effect of nitrite while addition of iron-chelating compounds enhanced the nitrite effect. Nitrite has the additional attributes of giving cured meats their pink coloration as well as acting as an antioxidant (Rhodehamel et al., 1992).

The presence of competitive microflora can allow the food processor to use lower levels of other preservative compounds such as nitrites in semipreserved meats. Lactic acid bacteria are generally added to the product along with a fermentable carbohydrate such as glucose or sucrose. Temperature abuse of the product promotes rapid growth of the competitive microflora and fermentation of the carbohydrate with a concomitant drop in the pH of the system. Some lactic acid bacteria also produce bacteriocins which have been shown to be inhibitory to *C. botulinum* under some conditions (Tanaka et al., 1985).

Thermal resistance of *C. botulinum* endospores.

Endospores of *C. botulinum* are of critical importance to food industries due to their very high thermal resistance. Spores of group I *C. botulinum* are much more heat-stable than those of group II. Consequently, most work involving thermal processing and D and

Z values has been done with group I organisms. A D-value is equal to the heat-treatment that results in a 90% reduction in the bacterial population of interest. A Z-value is the change in the temperature that results in a 10-fold change in the D-value. The minimum thermal process that is applied to low-acid ($\text{pH} > 4.6$), non-refrigerated, commercial foods is called a 12D process. This process theoretically reduces a load of 10^{12} viable endospores to 10^0 . Since the hypothetical 10^{12} spore load is much higher than any seen in the canning industry, such a process ensures that there are no viable *C. botulinum* spores remaining in the food (Rhodhamel et al., 1992). $D_{121^\circ\text{C}}$ values for group I spores have been reported to be 0.03-0.23 min (ICMSF, 1980).

The ability of group II organisms to grow at refrigeration temperatures is important due to the fact that many refrigerated foods do not receive a 12D thermal process but instead are pasteurized and then refrigerated. Because of this fact, endospores of group II organisms are usually tested for thermostability at 82°C . Non-proteolytic type B spores have reported $D_{82^\circ\text{C}}$ values of 1.5-32 min while other group II organisms such as type E strains and non-proteolytic type F strains are in the $D_{82^\circ\text{C}}$ 0.2-1.0 min range (Hauschild, 1989).

Distribution of *C. botulinum*.

The endospores of *C. botulinum* are found worldwide in soil samples and in freshwater and marine sediments (Rhodhamel et al., 1992). The distribution of in the United States is such that type A *C. botulinum* occurs more frequently in the western U.S. while type B occurs more commonly in the eastern U.S. Type E spores are often associated with marine and freshwater sediments.

II. The disease.

Foodborne botulism.

The disease botulism is classically associated with the consumption of preformed toxin in contaminated foods. Originally, blood-sausages were known to be a cause of the disease. The Latin word for sausage is *botulus*. The disease was first reported by van Ermengem in 1897 after an investigation of an outbreak in Belgium in 1895 (van Ermengem, 1979 translation). The causative organism was recovered from inadequately cured, unsalted ham and had caused 13 cases of botulism, 3 of which were fatal. The organism was named *Bacillus botulinus* by van Ermengem. van Ermengem showed that culture filtrates of this organism caused botulism in various animals and that the agent was heat labile and stabilized by acid. Since the time of van Ermengem, botulism has been associated with many other types of foods as well as with other disease etiologies. In the United States, the primary source of foodborne botulism is the consumption of improperly canned vegetables. Recently, the largest outbreaks of botulism in the United States have involved restaurants. In one the implicated food was improperly canned jalapeno peppers (Terranova et al., 1978); in another potato salad was implicated (Seals et al., 1981); and in yet another the toxin was formed in sautéed onions (MacDonald et al., 1985). Most of the foodborne outbreaks of botulism in the continental United States are caused by type A or B producing strains of *C. botulinum* while 32 of 44 outbreaks in Alaska since 1944 have been caused by type E *C. botulinum* (Wainwright et al., 1986). Type E *C. botulinum* is usually associated with fish or other marine-related foods such as dried and smoked fish, fermented whale blubber, and seal meat stored under seal oil (Wainwright et al., 1986).

Wound botulism.

A much rarer type of botulism is caused by *C. botulinum* contamination of a wound with subsequent growth of the organism and production of the toxin *in vivo* rather than ingestion of the preformed toxin. This type of botulism is termed wound botulism and is analogous to the disease caused by a close relative of *C. botulinum*, namely *C. tetani*.

Infant botulism.

* Infant botulism is currently the leading cause of botulism in the United States. This disease is caused by the ingestion of endospores and growth of *C. botulinum* in the infants gut with subsequent absorption of the toxin through the intestinal barrier. In the United States between 1975 and 1991 there were a total of 1013 cases reported to the CDC of which 480 were caused by type A *C. botulinum* and 522 were caused by type B *C. botulinum* (Rhodehamel et al., 1992). In a study of 336 patients with infant botulism, correlation has been demonstrated between toxin from stool samples and the presence of culturable *C. botulinum* (Hatheway and McCroskey, 1987).

III. The etiological agent.

C. botulinum toxin complex.

Toxins of the different C. botulinum serotypes are usually produced in culture as aggregates of neurotoxin and other non-toxic proteins associated into a polypeptide complex (Schantz, 1964; Sugii and Sakaguchi, 1975; Kozaki et al., 1974; Miyazaki, et al., 1977; Kitamura et al., 1969; Ohishi and Sakaguchi, 1974; Yang and Sugiyama, 1975; Nukina et al., 1987). These toxin complexes vary in size from ca. 900,000 daltons for type A LL toxin complex (Schantz, 1967) to ca. 300,000 daltons for the type B M complex (Kozaki et al., 1974) and type E complex (Kitamura et al., 1969), to 235,000 daltons for type F M complex (Ohishi and Sakaguchi, 1974). Descriptions of the sizes of toxin complexes has been confusing since various methods have been used in these determinations. Molecular weight determinations of the various sized complexes in the 1960's through the 1970's was done with ultracentrifugation and sizes were expressed in terms of Svedberg units. The results of some of these studies are shown in Table 1 (adapted from Sugiyama, 1980). Toxin complexes are described as M for medium, L for large and LL for very large. According to Sugii and Sakaguchi (1977), during culture the proportion of one toxin complex vs. another is dependent on the growth medium and conditions. A type B culture grown in the presence of 1 mM Fe^{+2} produces an equal proportion of L and M complexes while the same culture grown in the presence of 10 mM Fe^{+2} produces predominantly M complex.

Table 1. Molecular sizes of various *C. botulinum* toxin complexes.

| Toxin type | Sedimentation coefficient | ca. M_r (kDa) |
|----------------------------------|---------------------------|-----------------|
| LL A | 19S | 900 |
| L A, B, D | 16S | 450-500 |
| M A, B, C ₁ , D, E, F | 10-12S | 235-350 |

Some of the non-toxic proteins associated with the various toxin complexes have hemagglutinating abilities (Sugiyama, 1980; Somers and DasGupta, 1991). In particular, non-neurotoxic fractions of the L complexes of type A, B, and D have been shown to have hemagglutinating activity. Hemagglutinin fractions isolated from the different serotypes show some serological cross-reactivity. Non-toxic fractions from type A and B serotypes cross-react (Goodnough and Johnson, 1993) as do non-toxic fractions from types E and F. The non-toxic fractions of types C₁ and D are antigenically identical as determined by Ouchterlony diffusion (Sakaguchi et al., 1974).

The non-toxic complexing proteins have been demonstrated to be essential for stabilization of the toxin during passage through the digestive tract (Ohishi and Sakaguchi, 1974; Sakaguchi et al., 1981). Pure neurotoxin has a peroral LD₅₀ about 100-10,000 times lower than that of toxin complex on a weight basis (Ohishi, 1984; Sakaguchi, 1983). Presumably, the complexing proteins protect the very labile toxin molecule from proteolytic hydrolysis and other means of inactivation by enzymes present in the gut and circulatory systems since the toxin and the complexing proteins are very stable in low pH environments. The relationship between the size of the toxin complex and the peroral

LD₅₀ is such that the larger the toxin complex the lower the LD₅₀ indicating that there is a protective effect occurring in the gut. The nontoxic proteins may also promote absorption of the neurotoxin across the intestinal barrier. Following absorption, the neurotoxin component occurs free in the lymph and blood of animals fed toxin complexes (Heckley, 1960; Sugii et al., 1977).

Analysis by SDS-PAGE has shown that type A toxin complex consists of seven different nontoxic proteins ranging in size from ca. 17,000 daltons to 118,000 daltons in association with a neurotoxic protein of ca. 147,000 daltons (Gimenez and DasGupta, 1993; DasGupta, 1980; Goodnough and Johnson, 1993, in press). Isolated type A toxin complex has a specific toxicity of 2.4×10^7 intraperitoneal LD₅₀/mg in 18-22g white mice. Specific toxicities of other *C. botulinum* toxin complexes are type B M complex- 4.9×10^7 LD₅₀/mg, type C₁ M complex- 3×10^7 LD₅₀/mg, type D M complex- 8×10^7 LD₅₀/mg, type E M complex- 1×10^7 LD₅₀/mg, type F M complex- 2.3×10^7 LD₅₀/mg (Sugiyama, 1980), and 8.9×10^6 /mg for type G (unpublished data).

C. botulinum neurotoxin.

The active neurotoxin of *C. botulinum* is a dichain molecule of ca. 150 kDa in molecular weight. The molecule is composed of two fragments or chains that are termed the heavy chain (Hc, ca. 100 kDa) and the light chain (Lc, ca. 50 kDa) that are connected by one disulfide linkage. The neurotoxin is synthesized by the organism as a single polypeptide and undergoes posttranslational processing termed nicking to generate the two separate chains by at least one protease (Figure 1) (Yokosawa et al., 1986; Krysinski and Sugiyama, 1981). The nicking event occurs in the culture fluid for proteolytic *C. botulinum* and through the activity of an exogenous enzyme such as trypsin in non-proteolytic strains (Yokosawa et al., 1986, DasGupta, 1990; Kozaki, 1985). Various sizes and properties of botulin neurotoxins are shown in Table 2.

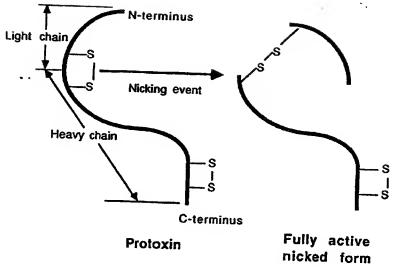


Figure 1. Activation of *C. botulinum* neurotoxin forming separate heavy and light chains.

Table 2. Properties of various *C. botulinum* neurotoxins (adapted from DasGupta, 1983; Smith and Sugiyama, 1988).

| Serotype | M_r (kDa) | | Extinction coefficient $E_{0.1\%}^{1\text{cm}}$ | pI | LD ₅₀ /mg protein |
|----------------|-------------|-------------|--|------|------------------------------|
| | Heavy chain | Light chain | | | |
| A | 93 | 52 | 1.63 | 6.1 | 1.05×10^8 |
| B | 101 | 53 | 1.85 | 5.25 | 1.14×10^8 |
| C ₁ | 98 | 53 | 1.42 | | 4.3×10^7 |
| D | 85 | 56 | | | |
| E | 100 | 55 | 1.63 | | 6×10^7 |
| F | 105 | 56 | | 5.7 | 2.5×10^7 |
| G | | | | | 2.4×10^7 |

Non-chromatographic methods

Crude purification of botulinum toxin was first demonstrated by Snipe and Sommer (1928) when they showed that 90% of the toxic material in a pure culture of *C. botulinum* could be recovered by addition of acid to pH 3.5. The first reported purifications of type A botulinum toxin that recovered a quantity of material of sufficient purity for crystallization was that of Lammana et al. (1946). This method utilized a step that included shaking the extracts of the acid precipitated material with chloroform. The method was further amended by Duff et al. (1957) who included precipitation of extracted toxin with ethanol as a means of removing nucleic acids. This method as modified by Schantz (1964) is the method that was approved by the U.S. Food and Drug Administration for production of type A toxin complex for use in humans. This method was selected over others because it is relatively simple and straight forward; it avoids exposure of the toxin to substances such as enzymes or resins from columns and it yields sufficient material for use on a commercial basis (Schantz and Johnson, 1992). This method is described in more detail in chapter III of this thesis, "Characterization of type A *Clostridium botulinum* toxin complex during purification."

Chromatographic methods

Column chromatography was first used in the purification of type A and B botulinum toxin by DasGupta and Boroff (1968). This method yielded purified neurotoxin from crystalline type A toxin complex using ion-exchange chromatography and showed for the first time that the specific activity of purified type A neurotoxin was ca. 10^8 LD₅₀/mg. Anion-exchange on DEAE-Sephadex A50 (Pharmacia, Piscataway, NJ) and gel-filtration chromatography (Sephadex G-200, Pharmacia) were used in the early 1970s to purify

toxin from culture fluids without first crystallizing the toxin (DasGupta et al., 1970; Sugii and Sakaguchi, 1975; Ohishi and Sakaguchi, 1977). Others used gel-filtration to obtain partially purified type B toxin complex from culture fluids (DasGupta and Sugiyama, 1976). Type A neurotoxin is now generally purified according to the method of Tse et al. (1982).

With the introduction of different types of ion-exchange resins, cation-exchange was used to further purify neurotoxins that had been partially separated from the non-toxic proteins of the complex (DasGupta et al., 1970; Sugiyama et al., 1974; DasGupta and Sathyamoorthy, 1984; DasGupta and Sugiyama, 1976). SP-Sephadex C50 (Pharmacia) is the most commonly used matrix for this type of chromatography. This type of ion-exchange matrix is not suitable for initial separation of the neurotoxin from the non-toxic complex proteins because of the fact that pH values under 7 are utilized at which the complex is stable. Neurotoxin is separated from most complex proteins at slightly alkaline pH values (DasGupta and Boroff, 1968). An alternative method of separating neurotoxin from complex proteins is through the use of affinity chromatography (Moberg and Sugiyama, 1978). Toxin complex adsorbs to p-aminophenyl- β -D-thiogalactopyranoside-Sepharose at acidic pH. The neurotoxin is then eluted from the column at alkaline pH with sodium chloride. Type E neurotoxin has been successfully purified from the non-toxic proteins of the complex using high-pressure liquid chromatography (Schmidt and Siegel, 1986). Fast protein liquid chromatography has been used to purify types A, B, and E neurotoxins on anion and cation exchange columns (Woody and DasGupta, 1988).

Biochemical characterization of botulinum neurotoxins.

The neurotoxin of type A botulinum toxin is a protein of ca. 147 kDa comprised of 1295 amino acids (Binz et al., 1990). Two peptide bonds may be cleaved during processing releasing a tetrapeptide (DasGupta and Dekleva, 1990). The nicking occurs approximately

one-third of the distance from the N-terminus of the protoxin molecule removing four amino acids and generates the neurotoxic Hc and Lc connected by at least one disulfide bridge. In type A neurotoxin, the Hc and Lc have molecular weights of ca. 93,000 and 52,000 daltons, respectively (Gimenez and DasGupta, 1993) which corresponds to the predicted molecular weights from the nucleotide sequence of the neurotoxin gene (Binz et al., 1990; Niemann, 1991). The two chains are connected by a disulfide bond between cysteine residues 430 and 454 (Gimenez and DasGupta, 1993; Binz et al., 1990). Reduction of this disulfide bond by sulfhydryl reducing agents such as dithiothreitol or mercaptoethanol generates the separate Hc and Lc. These individual fragments are non-toxic alone but recover toxicity when recombined under appropriate conditions (Maisey et al., 1988). There is a substantial degree of homology between the Hc and Lc chains of the different botulinum serotypes as well as to tetanus toxin (Niemann, 1991; Whelan et al., 1992). The overall percentage identity between the amino acid sequences of the genes for the Lc from tetanus toxin and Lc from botulinum toxin types A, B, C1, D, and E range from 32 to 51%. There are regions in the various neurotoxins that are strictly conserved many of which probably contribute to biological function. In the H chain, 110 amino acids of ca. 845 total are strictly conserved, and in the L chain 68 amino acids of the ca. 442 total are conserved (Niemann, 1991; Whelan et al., 1992). The availability of the complete amino acid sequences has also revealed the presence of a highly conserved region of hydrophobicity in the H chain, possibly involved in membrane fusion and transport, and also a zinc binding motif in the L chain of serotypes A, B, D, E, and F putting these neurotoxins into the category of zinc metalloendoproteases (Schiavo et al., 1992a; Schiavo et al., 1992b).

Mechanism of action of *C. botulinum* neurotoxin.

The target of botulin neurotoxin is the presynaptic junction of motor neurons. The proposed three step mechanism involves binding of the neurotoxin to the target cell surface, uptake or translocation across the cell membrane and internalization via endocytosis, followed by the inhibition of release of the cholinergic neurotransmitter (Simpson, 1981, Niemann, 1991). The receptor itself has not been identified but may involve polysialylated gangliosides or a specific protein (Niemann, 1991). Niemann also suggested that there are at least two different types of receptors with differing affinities. The low-affinity gangliosides are present in abundance (Critchley, et al., 1988) while the presence of a high-affinity protein receptor that is comparatively rare has been suggested by others (Yokosawa et al., 1989; Evans et al., 1985). This work was done with rat-brain and spinal cord membranes under physiological conditions. The existence of high-affinity protein receptors has been questioned, however, since pathological effects of botulin neurotoxin on brain tissue have never been observed *in vivo* after systemic administration (Niemann, 1991). The binding observed *in vitro* may be due to exposure of protein receptors to the heavy chain of the neurotoxin during preparation of the membranes (Niemann, 1991).

The Hc in all cases is believed to be the binding and internalization trigger for the toxin molecule while the Lc catalyzes the inhibition of synaptic vesicle release when internalized by the target cells (Simpson, 1989). Lc is not taken up by neuronal cells alone and Hc has no neurotoxic effects without being linked to the Lc (Maisey et al., 1988). Chimeric toxins have been created using the Hc of botulinum toxin and the Lc of tetanus toxin that show binding to the neuronal target of botulin toxin, and paralysis by a mechanism similar to tetanus toxin (Weller et al., 1991). Binding to receptors is believed to be mediated by the C-terminal half of the Hc (Niemann, 1991; Kozaki et al., 1989; Moteucucco, 1986), while the N-terminal half of the Hc facilitates passage of the Lc through

cellular membranes (Niemann, 1991; Blaustein, 1987). Montecucco (1986) presented a model for binding of botulinum neurotoxins to neuronal membranes. He proposed that the initial recognition site of the toxin on the neuronal membrane is a ganglioside of the G_{1b} series. The binding of botulinum toxins and tetanus toxin to gangliosides exclusively is not substantiated by certain pieces of evidence that show binding is trypsin sensitive and that radiolabelled tetanus heavy chain can not be displaced by unlabeled heavy chain. This evidence supports the hypothesis that there is a higher-affinity binding site on the neuronal membrane. Such a binding site could be a protein closely associated with the G_{1b} gangliosides. That the high-affinity binding site is not bound directly by the heavy chain of the neurotoxin could be explained by a conformational change in the heavy chain when bound first to the lower-affinity ganglioside which allows the heavy chain to recognize the high-affinity protein receptor.

The specific substrate of proteolytic cleavage by Lc of botulinum toxin type B and tetanus toxin was shown to be a specific isoform of synaptobrevin, synaptobrevin-2 (VAMP for vesicle associated membrane protein), an integral membrane protein of small synaptic vesicles (Schiavo et al., 1992b). VAMP is anchored to vesicles by a hydrophobic C-terminal tail structure while the remainder of the molecule remains exposed in the cytosol. Schiavo et al. (1992b) used highly purified synaptic vesicles from rat cerebral cortex to show that type B neurotoxin and tetanus toxin cleaved synaptobrevin-2 (VAMP) at Gln-76-Phe-77. Another VAMP, synaptobrevin-1 (VAMP-1), is virtually identical to VAMP-2 except that VAMP-1 has a valine residue substituted for the Gln-76 and it was resistant to cleavage by the endopeptidases neurotoxin B and tetanus toxin. The rate of cleavage of VAMP-2 correlated well with the inhibition of neurotransmitter release from *Aplysia* neurons indicating that the vesicle protein that was being cleaved was related to acetylcholine release (Poulain et al., 1989). Incubation of type B neurotoxin with VAMP-2 and a synthetic peptide that contained the cleavage site of VAMP-2 delayed the proteolysis

of VAMP-2. The next botulinum toxin serotype that was shown to cleave a synaptic vesicle protein was type F (Schiavo et al., 1993a). Type F neurotoxin was shown to cleave both isoforms of VAMP at a unique Gln-Lys peptide bond present in both VAMP isoforms. This site corresponds to Gln60-Lys61 of VAMP-1 and Gln58-Lys59 of VAMP-2.

The specific intracellular target of type D botulinum neurotoxin was shown to also be VAMPs. Type D neurotoxin cleaved both VAMP isoforms at a single site that corresponded to Lys61-Leu62 of VAMP-1 and Lys59-Leu60 of VAMP-2. These cleavage sites are one residue down from the target of type F neurotoxin (Schiavo et al., 1993b).

Type A and E botulinum neurotoxin were shown to have a different substrate than botulinum toxin types B, D, and F as well as tetanus toxin. Sollner et al. (1993) have shown that SNAP-25 (synaptosomal associated protein of 25 kDa) and VAMP are part of a multicomponent 20S protein complex proposed to facilitate vesicle docking and fusion. SNAP-25 was shown to be degraded by types A and E neurotoxin (Schiavo et al., 1993b; Schiavo et al., 1993c). The specific cleavage site was shown to be in the C-terminal region of SNAP-25. Using recombinant SNAP-25 the authors showed that the cleavage sites for A and E botulinum neurotoxins are separate and correspond to Gln197-Arg198 for type A neurotoxin and Arg180-Ile181 for type E neurotoxin.

The findings that botulinum toxin types B, D, F, and tetanus toxin have the same protein substrate which differs from that of toxin types A and E correlates well with the electrophysiological data that have placed the toxins in the same groups (Molgo et al., 1990).

V. Treatment of hyperactive muscle disorders with C. botulinum toxin.

History.

The use of type A C. botulinum toxin complex as a method of treating spastic muscle disorders was begun in the late 1970's by Drs. A. Scott and E. Schantz. The idea was to selectively paralyze hyperactive muscle groups by taking advantage of the mechanism of the toxin as an alternative to surgical procedures which involve mechanically severing nerves. Work began in earnest by the early 1980's using monkeys as models. After more than 20 years of collaboration between Scott and Schantz, crystalline type A toxin complex was licensed as an orphan drug in 1989 by the United States Food and Drug Administration. Approved treatments in patients 12 years of age and older include strabismus, hemifacial spasm, and blepharospasm (Schantz and Johnson, 1992). Since that time type A toxin has been used experimentally in the treatment of many other hyperactive muscle disorders. Some of these dystonias are summarized in Table 3. Dystonias are currently defined as "a syndrome of sustained muscle contractions, frequently causing twisting and repetitive movements of abnormal postures" (Schantz and Johnson, 1992). Treatment of spastic muscle disorders involves injection of nanogram quantities of the neurotoxin directly into the affected muscles. This creates a regional field of denervation and an overall reduction in muscle activity and pain (Borodic et al., 1991). Treatment duration varies with each indication but usually lasts for a period of several months.

Table 3. Uses of crystalline type A botulinum toxin in the treatment of spastic muscle disorders (adapted from Schantz and Johnson, 1992; Jankovic and Brin, 1991).

| <u>Condition</u> | <u>Symptom of disorder</u> |
|--|--|
| Strabismus | Crossed eyes |
| Blepharospasm | Uncontrollable blinking |
| Hemifacial spasm | Facial twitching and spasms |
| Eyelid disorders | Inward turning of eyelid |
| Spasmodic torticollis | Abnormal movement of head and neck |
| Oromandibular and lingual dystonias | Sustained mouth closure or lingual muscle contractions |
| Focal dystonias of the hand | Writer's cramp, musician's cramp |
| Spasmodic dysphonia | Uncontrollable vocal spasms |
| Neurogenic bladder | Abnormal urinary control |
| Limb spasticity including cerebral palsy | Occurs following strokes; other neurologic disorders |

Side effects of treatment with botulinum toxin.

The use of botulinum toxin in low dose applications (<20 LD₅₀) has not been shown to cause adverse effects. Large dose applications such as those used in the treatment of spasmodic torticollis (>100 LD₅₀) on a repetitive basis has caused a number of patients to develop neutralizing antibodies to the toxin (Jankovic and Brin, 1991; Scott, 1989). This is covered in more depth in chapter VI of this thesis. Other side effects from the use of botulinum toxin therapeutically include weakening and ptosis of nearby muscle groups due to the spread of the toxin from the point of injection. In treatment involving the muscles of the head and neck, one of the most prevalent adverse reactions is dysphagia or the inability to swallow. This has lead to upper airway obstruction in several patients (Borodic et al., 1991; Stell et al., 1988).

Properties of botulinum toxin for medical use.

In Figure 2, the SDS-PAGE electrophoretic pattern of the batch of type A toxin complex that was approved for use in humans (79-11) is shown. Lane 1 shows unreduced purified type A neurotoxin (molecular weight 145 kDa). Lane 2 (unreduced) shows the batch of toxin currently used medically (batch #79-11 produced at the UW-Madison, Food Research Institute in 1979 and stored at 4°C for 14 years). Lane 3 shows batch 79-11 reduced with 0.5% (w/v) dithiothreitol. Lane 4 shows molecular weight markers (from top to bottom in kDa) of 205, 116, 94, 66, 45 and 29. The molecular weights of the various toxin and nontoxic-complexing proteins present in lane 2 are as follows from the top of the gel (origin) in kDa: ca. 175, 147, 118, ca. 75, 50, 39, 35, 29, 22.8, 21.8, and 17. When 79-11 is treated with a disulfide reducing agent (lane 3), the Hc (93 kDa) and the Lc (52 kDa) of the toxin molecule are observed. Compared to other batches of toxin produced in our laboratory (see chapters III, IV, and V, this thesis), the batch 79-11 has deteriorated

substantially which may partly explain its relatively low specific toxicity (2-4 LD₅₀/mg, Botox® product insert) in the commercial product supplied to physicians.

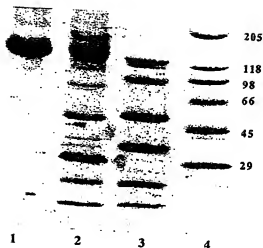


Figure 2. SDS-PAGE of purified type A neurotoxin and crystalline type A toxin complex batch 79-11 on a 8-25% acrylamide gradient gel. Lane 1, purified type A neurotoxin (unreduced); lane 2, batch 79-11 9 (unreduced); lane 3, batch 79-11 (reduced with 0.5 % w/v dithiothreitol); lane 4, molecular weight markers (rabbit myosin- 205 kDa, E. coli β -galactosidase- 118 kDa, rabbit phosphorylase b- 98 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, carbonic anhydrase- 29 kDa) (Sigma Chemical Co., St. Louis, MO). All lanes contained 4-6 μ g protein.

VI. Detection of *Clostridium botulinum* toxin.

Bioassay.

Detection of the toxin of *Clostridium botulinum* has been a concern since the time of van Ermengem in the late 1800's due to its presence as a contaminant in improperly handled foods. The classic method of detection is bioassay in animals. This method involves the injection of culture supernatant into a susceptible animal model and observing the animal for overt signs of botulism with an endpoint of death. The qualitative method of detection involves intraperitoneal injection of 0.1-0.5 ml of culture supernatant into 18-22 g white mice followed by observation for signs of botulism for the next four days. Animals that show signs such as labored breathing and a contraction of the oblique muscles of the abdomen within 2-24 h will usually die within the four day period. Serotype of culture supernatants that show the presence of botulinum toxin are determined by mixing samples of the toxic fluid with monovalent antisera that are specific for a single botulin type. Confirmation of the presence of botulin toxin is given when a single serotype of antiserum neutralizes the toxic effect. This presumes that there is only a single serotype of toxin present in the sample. A sample could contain two different serotypes of toxin that were produced by either a mixed population of botulinogenic organisms or by a single *C. botulinum* strain as in the case of A_g producing strains. It is also possible that interfering substances from the sample cause death. In such cases, monovalent antisera will not protect the animal but dilution of the sample usually suffices to reduce this occurrence. Quantitation of botulinum toxin is done in terms of the amount of toxin needed to kill 50% of a population of test animals. This amount is termed 1 LD₅₀ and is a statistical measurement based on the number of animals in each test group and the susceptibility of the test animals to the toxin. Quantitation is usually done with 5-10 animals /dilution and the results plotted semilogarithmically with % death on the vertical axis and dilution on the

horizontal axis. The point at which the line connecting the % death at each dilution crosses 50% is the dilution that contains 1 LD₅₀/injection volume (Schantz and Kautter, 1978). From this curve the number of LD₅₀ in the original sample is calculated. This method is both expensive due to the animals used and time consuming as well. For these reasons other methods of detecting botulinum toxin have been devised.

An alternative method of determining the amount of active botulinum toxin in a sample is the intravenous time-to-death method of Boroff and Fleck (1966). This method involves injection of a sample into the tail vein of an immobilized mouse. The time-to-death is noted and converted to the number of intraperitoneal LD₅₀ using a standard curve prepared using the dilution to extinction method of Schantz and Kautter (1978). The method is fast and relatively accurate ($\pm 20\%$). Drawbacks to this method are the preparation of the standard curve and the need for a higher level of technical expertise. The method is also applicable only when the amount of toxin in the sample falls within the linear portion of the standard curve (1×10^6 - 5×10^3 LD₅₀/ml of type A toxin).

Serologic methods.

Antibodies raise against detoxified botulinum toxin specific to a single serotype have been used in a number of ways to detect and quantitate the amount of toxin in given sample. A few of these serologic methods are presented below.

Passive hemagglutination/reversed-passive hemagglutination.

Passive hemagglutination involves formalin treated sheep red blood cells that are conjugated to toxin molecules or fragments of toxin molecules while reversed-passive hemagglutination uses antibodies specific to individual toxin serotypes conjugated to blood cells (Gordon et al., 1958; Johnson et al., 1966; Sakaguchi et al., 1974). The assay is done by adding dilutions of the corresponding toxin sample or antitoxin sample to the

sensitized sheep red blood cells in a microtiter plate and examining the plate for agglutination of the blood cells. It has been reported that the sensitivity of the reversed-passive hemagglutination assay using polyclonal IgG antibodies purified by affinity chromatography and conjugated to sheep red blood cells was as low as 8-10 LD₅₀/ml for types A and B toxins (Sakaguchi et al., 1974). The method of reversed-passive hemagglutination suffers from some of the following problems that are not uncommon to other antibody based botulinum toxin assays: 1) the assay does not distinguish between biologically active toxin and inactive toxin; 2) cross-reaction is seen between different serotypes presumably due to the quality of the coupled antibodies; and 3) hemagglutination is a characteristic of some of the botulinum toxin complex proteins, specifically A, B, and F, and may be a characteristic of other components of the sample being tested.

Immunodiffusion.

Immunodiffusion has been used to detect botulinum toxins in a manner analogous to the Ouchterlony assay (Crowle, 1958; Wadsworth, 1957) whereby toxin and antitoxin specific for a given serotype of toxin are allowed to diffuse toward each other through agar or agarose. At the point at which the two preparations converge, a visible line of immunoprecipitate is formed (Vermilyea et al., 1968). There are variations on the technique using microscope slides (Vermilyea et al., 1968) as well as capillary pipets partially filled with antitoxin mixed with agar (Mestrandrea, 1974). This method suffers from a lack of sensitivity (on the order of 300-500 LD₅₀/ml) as well as not being able to distinguish between active and inactive toxin.

Radioimmunoassays.

Radioimmunoassays based on the ability of an antibody to recognize a specific serotype of botulinum toxin were first used in 1973 (Boroff and Shu-Chen, 1973). The method commonly uses ^{125}I labeled toxin and antibodies specific for a given serotype. Unlabeled toxin present in a sample competitively binds the fixed amount of antiserum present. Hence, the more toxin present, the lower the radioactivity recovered bound to antibodies. One difficulty encountered is the fact that inactive toxin may be present and give too high an estimation of the amount of toxin present (Betley and Sugiyama, 1979). This noncorrelation between toxicity and antigenicity is the major drawback in all serological quantitations of botulinum toxin. Another obvious drawback to radioimmunoassays is the use of radioactive isotopes and the difficulties encountered in handling and disposing of them.

Enzyme linked immunosorbent assays.

ELISAs have been used for the detection of botulinum toxin since the early work of Notermans et al. (1978) in which a tube ELISA was described. The various ELISA methods use an antibody or series of antibodies specific for a given toxin serotype to capture and label the toxin. Typically in a double-sandwich type ELISA a tube or microtiter plate is coated with a capture antibody such as horse anti-botulinum type A. The sample is bound by the capture antibody and the toxin then labeled with another antibody specific to the toxin but from a different source than the first antibody. A third antibody labeled with a chromogenic enzyme is added that is specific for the second antibody, and color developed by addition of substrates for the chromogenic enzyme.

The major difficulty encountered in using the ELISA method for detection of botulinum toxin is the cross-reaction seen between different serotypes of toxin (Goodnough et al., 1993; Notermans et al., 1978). This cross-reaction is due to the

presence of antibodies that react to epitopes common to more than one serotype of toxin or to epitopes common to non-toxic complex proteins that contaminated the toxoid (Somers and DasGupta, 1991). There are numerous regions of homology that are conserved between the individual serotypes of toxin (Niemann, 1991; Whelan et al., 1992; Tsuzuki et al., 1988). The solution to the first difficulty, namely, contamination of the toxin antigen with non-toxic proteins from the complex can not be avoided entirely. However, neurotoxin preparations for use in antibody production can be made that are purified to the extent allowed by modern chromatography. The difficulty arising from the presence of a subpopulation of antibodies reacting to conserved regions of different toxin serotypes in a polyclonal pool has been addressed by the use of monoclonal antibodies (Kozaki et al., 1986; Gibson et al., 1988; Ferreira et al., 1990). An additional difficulty in the use of ELISAs for detection of botulinum toxin is the fact that the detection levels for most of the assays are in the range of 20-10,000 LD₅₀/ml (Notermans et al., 1978; Modi et al., 1987; Goodnough et al., 1993; Gibson et al., 1988). A method using snake venom as part of an amplification system to detect botulinum toxins has recently been published that claims to be as sensitive as the mouse bioassay detecting ca. 10 pg of neurotoxin (Doelgaest et al., 1993).

Others.

The polymerase chain reaction (Saiki et al., 1988) has been successfully used for detection of Clostridium botulinum in culture media (Szabo et al., 1992). This method used a set of primers specific for the type B toxin gene. The method detected as few as 100 fg of the target DNA (ca. 35 cells). The major drawback to this method is the fact that it can not distinguish between the organism and the etiologic agent, the toxin. The organism may be present without producing toxin as in the case of the dormant endospores. Its sensitivity is likely to be less in foods.

VII. Lyophilization.

Lyophilization is the method preferred to stabilize toxin for commercial supply to physicians. Freeze-drying or lyophilization, is a process in which the solvent is first frozen and then removed by sublimation in a vacuum environment (Pikal, 1990). The solvent is, usually water and is frozen in commercial freeze-dryers at a temperature of ca. -40°C . When the frozen product has solidified, the drying chamber is evacuated and the shelf temperature raised slowly to initiate the sublimation of the ice crystals. The water that is sublimed is removed from the drying chamber and collected on low temperature condensers (-60°C). The first stage of drying is termed primary drying and typically removes 50-80% of the water present. The water remaining in the product after primary drying is removed during secondary drying. Secondary drying is typically done at elevated shelf temperatures ($0-25^{\circ}\text{C}$) in order to remove the water remaining in the amorphous solid.

Freezing the water in a solution concentrates solutes in the solution to the point where many of them crystallize. Those that do not crystallize are transformed into a rigid glass when the system is brought below the glass transition temperature of the amorphous phase (Pikal, 1990). The glass transition temperature (T_g) is the point at which water in the mixture ceases to form ice crystals during freezing at least on a realistic time scale. This unfrozen water is characterized by the T_g and its water content. The properties of the amorphous mixture change at the T_g . Above the T_g , the water that is present in the amorphous mixture is free to diffuse and the viscosity of the material drops. The water that is present is free to participate in various chemical reactions such as deamidation, aggregation, and peptide bond hydrolysis which are all dependent on free water. Below the T_g , the mixture of water and glass is more rigid and diffusion rates are extremely slow on the order of $\mu\text{m}/\text{year}$ (Franks, 1990).

Crystallization of the water occurs after supercooling of the solution to $10-15^{\circ}\text{C}$ below the equilibrium freezing temperature. This temperature varies depending on the

solutes, the shelf temperature, and on the presence of particulate material to serve as ice nucleation sites. The degree of supercooling determines the size of the crystals and therefore is intimately associated with the structure of the freeze-dried product. The higher the supercooling, the smaller the ice crystals and the smaller the pores in the amorphous solute matrix. Smaller pores have a larger surface area than larger pores and so facilitate both primary and secondary drying.

Primary drying is a process of mass transfer from the frozen vials to the condenser of the freeze-drier. For sublimation of each gram of ice, ca. 1,000 liters of water vapor passes through the partially dried cake of material in each vial (Pikal, 1990). The rate of sublimation is dependent on the difference in pressure between the water vapor in the frozen product and the condenser of the chamber. The resistance to collection of the water vapor is dependent on vial size and shape, stopper configuration, and resistance across the partially dried cake. The first two can easily be controlled while the resistance to water vapor escape through the dried product can be controlled by the freezing process and the degree of supercooling of the solution. Temperature of the material is slowly raised during primary drying which also contributes to the rate of sublimation. Primary drying ends when all of the ice in the vials has been removed. The partial pressure of water vapor present in the drying chamber is nearly equal to the total during primary drying but drops sharply at the end of primary drying.

Secondary drying begins after the ice in the vials has been removed and continues until the final percent moisture levels have been reached. For non-protein systems this level may be less than 1% while protein products typically contain up to 4% water. This difference is primarily due to the differences in formulation with protein products using higher levels of cryoprotectants such as carbohydrates which have high levels of water of hydration (Franks, 1990).

Excipients used in freeze-drying

Compounds added to a solution to be lyophilized in addition to the active components are termed excipients. Excipients may consist of bulking agents to prevent "blowout" of the product. Blowout can occur when solutions with very low % solids are freeze-dried. The water vapor from sublimation can carry pieces of the freeze-dried material out of the vial in such cases. Buffers are frequently used which contribute to the overall % solids of the solution. Some products have salts such as sodium chloride added to yield isotonic solutions when reconstituted and still others have compounds such as arginine added to increase the solubility of the active component. Protein pharmaceuticals typically have a lyoprotectant added to stabilize the active compound during the freeze-drying cycle and subsequent shelf-life. Some of the compounds used for stabilization of protein pharmaceuticals include dextran, polyvinylpyrrolidone, polyethylene glycol, ficoll, gelatin, serum albumin, hydroxyethyl starch, trehalose, sucrose, lactose, arginine, glycine, and mannitol. The addition of inert proteins to a formulation containing pharmaceutical proteins of interest has been shown to stabilize the active moiety in solution and during lyophilization (Schantz, 1964; Schantz and Kautter, 1978; Goodnough and Johnson, 1992). However, the material usually has a relatively low shelf-stable temperature. In the case of botulinum toxin, the storage temperature for the commercial product is $<10^{\circ}\text{C}$. This is presumably due to the low T_g of the freeze-dried material. This issue is further addressed in Chapter IV of this thesis. Addition of polyhydroxylated compounds such as carbohydrates has been shown to provide a more shelf stable freeze-dried product than the corresponding product lacking the carbohydrate (Colaco et al., 1992; Mouradain et al., 1984; Roser, 1991; Franks et al., 1991). The mechanism by which carbohydrates such as trehalose stabilize protein molecules is unknown. One possibility is that by raising the glass transition temperature of the freeze-dried material, the storage temperature of the material is correspondingly elevated. It is also possible that the carbohydrate is somehow

replacing the water molecules that are involved in maintaining the tertiary structure of the molecule during lyophilization. Such an idea is termed the water replacement hypothesis and was proposed by Saenger (1989) and Otting et al. (1991). Others (Levine and Slade, 1938; Franks et al., 1991) have argued that the tendency for carbohydrates to form glassy states does not allow the molecular movement needed for the protein of interest to undergo degradative reactions.

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CHAPTER III

Colony immunoblotting of Clostridium botulinum types A, B, and E

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Abstract

Neurotoxin produced by colonies of Clostridium botulinum types A, B, E and toxigenic Clostridium butyricum bound to nitrocellulose was detected by an enzyme-linked immunoassay procedure. The procedure used serotype specific rabbit IgG as the primary antibody and goat antirabbit IgG antiserum labelled with alkaline phosphatase as the marker to visualize immobilized neurotoxin from individual colonies. The method differentiated the colonies based on serotype of neurotoxin produced and by the amount of neurotoxin produced by individual colonies on the same agar plate. Specificity of primary antibodies used was improved by adsorption of cross-reacting heterologous antibodies.

Introduction

The technique of using specific antibodies for the detection of immobilized proteins of interest has been described extensively since 1979 (Towbin, 1979). The more common techniques involve transfer of proteins from polyacrylamide-gels to a solid support such as nitrocellulose using an electrical current (Bitner et. al., 1980). The transferred proteins are then probed with antibodies specific for the protein of interest. In contrast to this electroblotting technique, we have been using for sometime in our laboratory a colony-blotting procedure for the detection, differentiation, and estimation of the varying toxin titers of the individual Clostridium botulinum colonies of types A, B, and E as well as two toxigenic Clostridium butyricum strains. The method uses alkaline phosphatase as the enzyme marker (Mason, 1978) and nitrocellulose as the solid support.

The potential uses of this technique include the screening of various ingredients in food products to determine the C. botulinum spore load prior to formulation as well as the efficacy of the subsequent heat-treatment of food products. Temperature abuse as well as adequacy of the total anti-microbial system could be monitored by simply using selective media followed by blotting. The follow-up procedure must include a more sensitive toxin assay system such as the mouse bioassay. The colony-blotting technique could potentially find use in the screening of products which do not receive a heat treatment to determine if there are C. botulinum spores present and at what levels. The colony blotting assay has found use in our laboratory as a convenient method of screening large numbers of potential mutants for toxin production.

Organisms.

Clostridium botulinum type A strains were Hall A, 73A, 90A, 109A, and 62A. *C. botulinum* type B strains were Okra B, Lammana B, 32B, 7949B, 113B, 213B, and 169B. *Clostridium sporogenes* strains 4411 and PA 3679 were used as the negative controls in the type A and B blotting tests. Type E *C. botulinum* strains were Alaska E, 5545E, and Iwarii E. All *C. botulinum* and *C. sporogenes* strains were from the Food Research Institute culture collection. *Clostridium butyricum* strains 5839 and 5521 produce a neurotoxin very similar to the botulinum type E toxin molecule (Gimenez and Sugiyama, 1988a). These toxigenic strains were obtained from Dr. C. Hatheway, Centers for Disease Control, Atlanta, GE. Non-toxigenic *C. butyricum* strains 19398 from the American Type Culture Collection and 1024 from the Food Research Institute collection were used as negative controls in the type E blotting procedure.

Culture media.

Colonies for the blotting tests were usually grown on TPGY agar (5% trypticase peptone, BBL; 0.5% bacto-peptone, Difco; 0.4% glucose; 0.2% cysteine-HCl; 0.1% yeast extract, Difco; 2.0% bacto-agar, Difco; pH 7.4) which supports growth of all test strains. In some cases, the three antibiotics of CBI agar (*C. botulinum* isolation medium (cycloserine, 250 mg/l, sulfamethoxazole, 75 mg/l, trimethoprim, 4 mg/l) (Dezfulian et al., 1981) were added to TPGY to make the medium selective for *C. botulinum* types A and B as well as some strains of type E.

C. butyricum colonies were sometimes grown on a minimal medium consisting of 1.0% glucose, 0.1% $(\text{NH}_4)_2\text{SO}_4$, 4.0% salts solution (2mM CaCl_2 , 1.7mM MgSO_4 , 5.7mM K_2HPO_4 , 7.4mM KH_2PO_4 , 120mM NaHCO_3 , 34mM NaCl), 0.4% resazurin

solution (250 µg/ml), 0.4% biotin (12.5 µg/ml), 0.1% thiamine-HCl (1 mg/ml), 0.05% cysteine-HCl, and 0.5% NaHCO₃). This GMBT medium is a modification of the GMB medium described in the VPI Anaerobe Manual, 4th edition (Holdeman, Cato, and Moore, eds.).

Antiserum.

Antitoxins for type A, B, and E botulinum toxin serotypes were raised in rabbits. Type A toxin was purified by the method of Tse et al. (1982), type B toxin by the method of DasGupta and Sugiyama (1976), and type E toxin by the method of Gimenez and Sugiyama (1987). These preparations were considered to be pure toxin samples since they showed only the approximate 150 kDa protein when electrophoresed in polyacrylamide gels without being treated with a disulfide reducing agent. When disulfide bonds were reduced with mercaptoethanol (1% w/v) or dithiothreitol (0.5% w/v), the preparations showed the characteristic ca. 100 kDa heavy chain and the ca. 50 kDa light chain.

The toxins were converted to toxoids by dialysis against 50 mM sodium phosphate buffer, pH 8.0 with 0.4% formalin for 14 days at 30°C. Most of the unreacted formalin was removed by dialyzing the toxoids against 65 mM sodium phosphate buffered saline (PBS), pH 7.4. The toxoids were homogenized in equal volumes of complete Freund's adjuvant (Difco Laboratories, Detroit, MI). The mixture was injected subcutaneously into rabbits in volumes containing 200-300 µg of toxoid. Each rabbit was then given a SC injection of ca. 100 µg of native toxin (ca. 10⁷ mouse 50% lethal doses) on days 37, 38, 39, 44, 45, 46, 51, 52, and 53. The rabbit was then bled by cardiac puncture on about day 60.

The serum (ca. 50 ml/rabbit) was dialyzed against 20mM Tris-HCl, pH 8.5 and applied to a Sepharose CL4B-Protein A column (1.6 cm x 22 cm) equilibrated in the same buffer at a rate of 30 ml/hr. After loading the crude serum, the column was washed with 5

column volumes of the loading buffer followed by a similar volume of 50 mM citrate/50 mM NaCl, pH 7.0. IgG bound to Protein A was eluted in a single protein peak when the buffer was changed to 50 mM citrate/50 mM NaCl, pH 3.0. The IgG fractions were pooled as soon as practical and the pH adjusted to ca. 7.0 with 1.0 M Tris-HCl, pH 9.0. The antitoxin was then dialyzed against 65 mM PBS, pH 7.4, and stored at 4°C in a final concentration of 25% glycerol.

Adsorption

The type A, B, and E antitoxins were type specific in toxin neutralization tests so that type A antitoxin did not neutralize type B or E toxin, type B antitoxin did not neutralize type A or E toxin, while type E antitoxin did not neutralize type A or B toxin. However, some *in vitro* serological procedures with the type A and B antitoxin types did cross-react to some extent with the heterologous toxin. In the past, these cross-reactions have made the distinction between toxin serotypes difficult and unreliable using the immunoblotting procedure. This cross-reaction is most likely due to the presence of small amounts of non-toxic complex proteins including hemagglutinating proteins in the antigenic preparations. Some of these non-toxic proteins have epitopes common to all of the toxin serotypes (Somers and DasGupta, 1992). In order to minimize these cross-reactions an adsorption procedure was developed. The procedure involves the addition of whole cells and concentrated crude toxin preparations of the heterologous toxin types to the IgG antiserum preparations of type A and B. The type E antiserum preparation did not cross-react to the extent of the A and B antiserum and was not adsorbed.

The strains used as adsorbants for type A antitoxin were *C. botulinum* strains 113B, 213B, and Okra B. Type B antitoxin was adsorbed with *C. sporogenes* strain 4411, and *C. botulinum* Hall A. Toxin concentrates were the precipitate formed when 450 ml of a 4 day TPGY culture was adjusted to pH 3.5 with 1N HCl. This was a modification

of the method of Tse, et. al. (1982). The precipitate was collected by centrifugation and suspended in ca. 5 ml of 65mM PBS, pH 7.4. Cells for use in the adsorption procedure were grown separately for 24 hr at 37°C in 50ml of TPGY broth, pH 7.4, collected by centrifugation, and washed three times with sterile 0.85% physiological saline. The separate strains for the respective antitoxins were combined and resuspended in ca. 5 ml of saline.

After combining crude toxin/whole cell preparations, a known number of international units (1 IU for types A and B = 10,000 mouse LD₅₀; 1 IU for type E = 1,000 mouse LD₅₀) of heterologous antiserum was added to ca. one-third of the total volume of adsorbant and allowed to react at room temperature for 30 min to 1 h. The mixture was then centrifuged at 12,100 x g for 20 min. The supernatant was reabsorbed twice in the same fashion. After the final adsorption, the antiserum was dialyzed against 20mM Tris-HCl, pH 8.5, and reapplied to the Protein-A column. The column was then extensively washed with the loading buffer (30-50 column volumes) to remove the heterologous toxin present. The adsorbed IgG fractions were then eluted as before with 50 mM citrate/50 mM NaCl, pH 3.0. The resulting antiserum preparation had ca. 10-100X less cross-reactivity than the parent antiserum. Each antiserum preparation was then retitrated against its homologous toxin type yielding values from 90 to 400 IU/ml for types A and B and 340 IU/ml for type E antiserum.

Colony blotting procedure.

The procedure used here is a direct immunostaining procedure of immobilized proteins. The complete immunosandwich is depicted schematically in Figure 1. Plates with surface inoculated colonies were grown anaerobically ≥ 48 h. Colonies were reasonably well isolated with ca. 100 colonies/plate maximum to ensure no overlap of antigen as toxin diffuses away from the colonies through the agar medium.

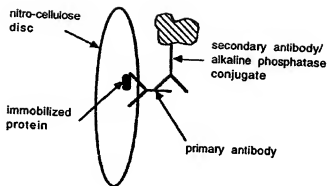


Figure 1. Schematic representation of immunoblotting of *Clostridium botulinum* colonies on nitro-cellulose discs.

The immobile phase used to bind the neurotoxins was nitrocellulose (NC) from Bio-Rad Laboratories, Inc., Richmond, CA. The NC came pre-cut into 82.5 mm diameter discs which were applied directly to the agar plates. The first NC disc was placed on top of the colonies by dragging the leading edge of the disc with forceps over the rim of the plate until the trailing edge dropped onto the plate. The NC became wetted slowly from the trailing edge toward the leading edge. This method of application ensured no air bubbles were trapped under the NC prohibiting contact with the plate and the colonies. The plate with the NC adhering to it was then inverted and held at room temperature for 2 h. The NC was removed from the plate by peeling it off in the reverse order of application. This NC disc could then be overlaid briefly onto a fresh plate and a replicate of the master plate made before the disc was blotted. This step required well dispersed colonies. A second disc could be overlaid onto the master plate at this time and the NC/plate incubated under the same conditions.

After removal from the master or duplicate plate, the colonies which adhered to the NC were removed with a gentle stream of distilled water. The NC was then placed into a blocking solution to completely saturate the unbound protein binding sites on the disc. The blocking solution consisted of 5% skim milk powder in TBS (10 mM Tris-HCl, 0.9% NaCl, 0.1% bovine serum albumin, pH 7.4) (Sigma Chemical Co., St. Louis, MO). Alternatively, 5% bovine serum albumin in TBS was substituted. These incubations were done in petri dishes with gentle agitation for 1 h at room temperature.

After 1 h incubation, the blocking solution was replaced with primary antiserum diluted with TBS + 1% bovine serum albumin, pH 7.4. Working concentrations of primary antiserum varied from one antiserum lot to the next. Optimum dilutions ranged from 0.8 IU/ml for some of the type A and B antisera to 10 IU/ml for the type E antiserum. Total volume used in the primary incubation step was 10.0 ml for a total of ca. 8-10 IU for

the type A and B blots and 100 IU for the type E blots. This incubation was carried out at room temperature for 1 h with agitation as before.

At the end of the primary antiserum incubation, the solution was decanted and the blot washed with TBS + 0.09% Tween 20, pH 7.4, to remove excess unbound antibodies. Three 75 ml washes were carried out in a large, flat-bottomed, plastic, storage container (ca. volume 1500 ml) with agitation for 10 min each. At the end of the third wash, a brief (2 min) rinse was done with TBS to remove excess detergent.

NC discs were transferred to clean petri dishes containing 10.0 ml of secondary antiserum (goat anti-rabbit IgG conjugated with alkaline phosphatase, Boehringer-Mannheim, Indianapolis, IN) diluted in TBS + 1% bovine serum albumin, pH 7.4, to a final concentration of 1:2000. The blots were incubated with agitation at room temperature for 1 h and then washed as before in TBS + 0.05% Tween 20 and 0.05% SDS, pH 7.4, for 10 min each. A brief rinse (2 min) with TBS was done to remove detergent which might interfere with enzymatic activity. After washing, the enzyme substrates were added in a 100 ml volume of 1 M Tris-HCl, pH 9.5. The substrates used were 5.6 mM 5-bromo-4-chloro-3-indolylphosphate (BCIP) plus 4.8 mM nitroblue tetrazolium (NBT) (both from Sigma). NBT was first made soluble in ca. 10 ml of boiling 1 M Tris-HCl, pH 9.5, and then added to the remaining 90 ml of 1 M Tris-HCl, pH 9.5. BCIP was dissolved in 100 μ l of dimethylsulfoxide and then added to the 1 M Tris-HCl, pH 9.5. After mixing, the substrates were added to the blots in the wash container and the purple color allowed to develop until the background began to appear (usually within 2-5 min)!

After full color development, the reaction was stopped by thoroughly rinsing the blots in distilled water and allowing them to air dry in the dark. The colored complex is light sensitive and will gradually fade upon exposure to sunlight.

The entire process of colony blotting may be stopped prior to any of the above steps by simply holding the blot in TBS. Blotting may then be resumed the following day with

no change in sensitivity. Alternatively, prior to development, the blots may be rinsed briefly in TBS and then frozen at -20°C for weeks to months without any change in reactivity.

SDS-PAGE

Polyacrylamide gel electrophoresis was done using the Bio-Rad Protean II system (Bio-Rad Laboratories, Richmond, CA). Linear 12.5% acrylamide gels were run at 20 mA constant current at room temperature according to the discontinuous system of Laemmli (1970).

Electrotransfer and immunoblotting of SDS-PAGE gels

SDS-PAGE gels of *C. botulinum* toxin samples were transferred to polyvinylidene difluoride membranes (PVDF) (Millipore, Corp., Bedford, MA) using the Bio-Rad Trans-blot Electrophoretic Transfer Cell. Transfers were made overnight at room temperature and 30 mA constant current in 10mM CAPS (3-(cyclohexylamino)propanesulfonic acid) (Sigma), 5% methanol, pH 10, following the procedure of Matsudaira (1987). Transferred proteins were visualized using the immunoblotting procedure described for colony blots. All incubations and washes were done in large flat-bottom (1500 ml) containers.

In order to determine whether the adsorption procedure for removal of cross-reacting antibodies from the type A and B antisera was effective, known amounts of crude culture of both toxin types in 10-25 μ l volumes were spotted side-by-side onto NC and the immunoblotting procedure performed as described for colony blotting using the antisera in question. The developed reactions showed that the reduction in cross-reaction was typically 10-100 fold. These dot-blots were also used to determine the limits of detection. Dilutions of each serotype of toxin were spotted onto the nitro-cellulose followed by the colony-blotting procedure. The limit of detection for the type A and B antiserum batches was in the range of 10-25 LD₅₀. Assuming a specific activity of 30 LD₅₀/ng for type A toxin complex, this is equivalent to 300-800 pg of toxin complex or 100-267 pg of purified type A neurotoxin.

Electrotransfer of type A toxin, type A toxin complex, and type B neurotoxin from a SDS-PAGE gel was done to establish whether type A antibodies reacted with type B neurotoxin. Figure 2 shows a 12% polyacrylamide gel which has been stained with 0.1% Coomassie brilliant blue R250 (Sigma) in 7.5% acetic acid and 25% methanol. Lanes 1 (unreduced) and 2 (reduced with 0.5% w/v dithiothreitol) contain purified type A toxin, lanes 3 (unreduced) and 4 (reduced) contain type A complex, and lanes 5 (unreduced) and 6 (reduced) contain purified type B toxin. Each lane was loaded with 10-15 μ g of protein.

Figure 3 shows the corresponding immunoblot from the gel depicted in Figure 2; it was developed after reaction with adsorbed type A IgG. Lanes are numbered in the reverse order of those in Figure 2. Purified type B toxin (lanes 1 and 2) is unreactive at this protein concentration while type A complex (lanes 3 and 4) and purified type A toxin (lanes 5 and 6) show reaction with the unreduced and the reduced toxin but not with the non-toxic proteins associated with the type A toxin complex. There is a faint signal in the

region of ca. 30kDa in both the reduced type A toxin samples which could be indicative of a breakdown fragment of the toxin molecule.



Figure 2. SDS-PAGE of *Clostridium botulinum* toxins. Lane 1, type A neurotoxin; lane 2, type A toxin neurotoxin (reduced with 0.5% w/v dithiothreitol); lane 3, type A toxin complex; lane 4, type A toxin complex (reduced), lane 5, type B neurotoxin; lane 6, type B neurotoxin (reduced). Each lane was loaded with 10-15 μ g of protein.

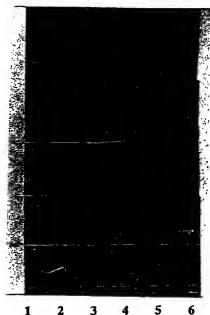


Figure 3. Immunoblot of proteins after electrophoretic transfer from the SDS-PAGE gel shown in Figure 2 using adsorbed type A IgG as primary antibody. Lane 1 (reduced) and lane 2 (unreduced) contain purified type B toxin, lane 3 (reduced) and lane 4 (unreduced) contain type A toxin complex, lane 5 (reduced) and lane 6 (unreduced) contain purified type A toxin. Each lane of the original gel was loaded with 10-15 μ g of protein prior to electrophoresis and electrophoretic transfer.

We found that of the three toxin types there were some strains which cross-reacted to a much greater extent than others. Hall A and 62A cross-reacted with the unadsorbed type B antiserum as did Okra B and 113B with the type A antiserum. The cross-reaction was not completely eliminated after adsorption as determined with the dot-blot procedure but was substantially reduced and did not interfere with the colony blotting procedure. Colonies of *C. sporogenes* 4411, PA 3679, and a *C. tetani* strain did not react with any of the adsorbed antisera.

A typical type A colony reaction is shown in Figure 4. It shows that the toxin which has diffused away from the colony is still bound to the NC and was detected. Because of this it is possible to overlay a second disc and develop it under different conditions using a different primary antiserum. This technique is shown in Figures 5, 6, and 7. Figure five shows the original plate which contained a mixed population of predominantly type A toxin producing colonies. The arrowheads indicate three colonies which produced type B toxin. The first disc overlaid on the plate was developed in type A specific antiserum (Figure 6) and the second disc in type B specific antiserum (Figure 7).



Figure 4. Typical type A *C. botulinum* toxin producing colony reaction on nitrocellulose using colony immunoblotting procedure.



Figure 5. TPGY agar plate containing a mixed population of *C. botulinum* type A and *C. botulinum* type B. The arrowheads indicate the type B colonies (from Goodnough et al., 1993).



Figure 6. Colony immunoblot of mixed population of *C. botulinum* types A and B toxin producing colonies using antitype A IgG as primary antibody (from Goodnough et al., 1993).



Figure 7. Colony immunoblot of mixed population of *C. botulinum* types A and B toxin producing colonies using antitype B IgG as primary antibody (from Goodnough et al., 1993).

Diffusion of toxin through the culture medium is also demonstrated in Figures 8 and 9. Figure 8 shows the size of the co-cultured *C. butyricum* strains 5839 (toxigenic) and 19398 (non-toxigenic) as grown on the minimal medium GMBT and the size of the reaction zone from the toxigenic strain (Figure 9). In all cases the plate and the blot are mirror images of each other. In Figure 9, the "negative" zones of non-toxigenic *C. butyricum* 19398 can be seen as lighter spots in the much darker "positive" zones of the toxigenic strain 5839. Individual colonies from the original plates or from a duplicate plate can then be reisolated.

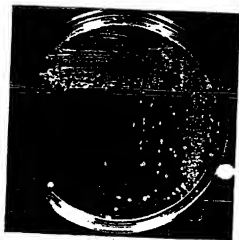


Figure 8. Mixed colonies of toxigenic *C. butyricum* (strain 5839) and nontoxigenic *C. butyricum* (strain 19398) on GMBT minimal medium.



Figure 9. Colony immunoblot using antitype E IgG as primary antibody. A mixed population of toxigenic *C. butyricum* (strain 5839) and nontoxigenic *C. butyricum* (strain 19398) was grown on GMBT minimal medium. The arrow indicates the position of a nontoxigenic *C. butyricum* colony in the darker background of toxin which has diffused from a nearby toxigenic *C. butyricum* colony.

Another use of this blotting procedure is that it can distinguish between high-titer and low-titer colonies on the same agar plate. Figure 10 shows a blot of colonies of C. botulinum 7949 B. The different intensity reactions are indicative of the toxin titers of the colonies. When isolated individually and grown in TPGY broth culture the colony giving the lighter reaction (indicated by the arrow) produced 10 fold less toxin/ml than the colony which gave the darker reaction.

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Figure 10. Colony immunoblot of *C. botulinum* type B (strain 7949) toxin producing colonies. The lighter reacting colony indicated by the arrow produced ca. ten-fold less toxin when isolated, grown in broth culture, and tested in the mouse bioassay (from Goodnough et al., 1993).

The sensitivity of the blotting procedure in our hands is on the order of 10-25 LD₅₀/spot. Assuming a relative toxicity for type A toxin complex of 3×10^7 LD₅₀/mg (Schantz, 1964), this detection limit is on the order of 300-800 picograms. This limit compares favorably with other enzyme-linked immunosorbent assays (Lyeryl, 1983; Kozaki, 1979; Laughon, 1984; Notermans, 1978). The limit of detection is critically dependent on the quality of the primary antiserum.

Cross-reaction of the different serological types of antisera could be due to the presence of antibodies to common non-toxic proteins associated with the different toxin serotypes or to similar epitopes being present on the different serotypes of toxin. These non-toxic proteins are conserved throughout the range of botulin serotypes (Somers and DasGupta, 1991) and any trace of carry over from the toxin purification to the antigenic preparation will generate cross-reactions in the blotting procedure. It is possible there are individual epitopes which are common to more than one serotype as well. The use of monoclonal antibodies may be of help to avoid these cross-reactions.

The technique of colony-blotting is an alternative method to the mouse assay for the detection of *Clostridium botulinum* neurotoxins. The traditional method of picking individual colonies followed by growth in broth medium and toxin testing in mice is time consuming and expensive. The standard mouse bioassay is still the method of choice when detection of very low quantities of toxin (≤ 10 LD₅₀) is necessary. However, for some laboratories without the facilities to properly house animals for toxin testing, this method may be useful. An additional use of the colony blotting procedure is in detection of toxin mutants in a population of mutagenized *C. botulinum*. This has been used in our laboratory in conjunction with transposon mutagenesis (Lin and Johnson, 1991) of *C. botulinum* type A. It may be possible to detect so called leaky mutants in regulation studies

of toxin formation using the colony blotting approach which is much easier than screening individual colonies for toxin titer.

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CHAPTER IV

Characterization of type A Clostridium botulinum toxin complex during purification

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Abstract

Purified type A *Clostridium botulinum* neurotoxin is used for the treatment of spastic muscle disorders due to the fact that it causes a flaccid paralysis in specific muscle groups when very dilute solutions of the toxin are injected. The toxin for medical use is produced by the Hall A strain as part of a complex of at least six other proteins and is purified by a series of precipitations and crystallizations. In this study the toxin complex was examined at each step in the purification for total toxicity, percent solids, optical density at 260 and 278 nm, specific toxicity, and for the presence of ribonucleic acids. The results show that the procedure is variable and dependent to a large degree on the individual components making up the growth medium indicating that nutrition and regulation affect toxin quality. RNA associated with the purified toxin complex was found at a level of 0.3% and electrophoretic analysis indicated that particular species of RNA were not present in the complex.

Introduction

Clostridium botulinum is a heterologous group of gram positive, obligately anaerobic, endospore forming rod-shaped organisms that have in common the production of a characteristic proteinaceous neurotoxin. There are currently seven known serotypes of neurotoxins (A, B, C₁, D, E, F, and G). These neurotoxins are most commonly known as being the cause of the food borne intoxication botulism (Sugiyama and Sofos, 1988). These proteins are among the most potent neurotoxins known with lethal doses on the order of 1 ng/kg for most mammals including humans (Schantz and Johnson, 1992). The mode of action of the neurotoxin is such that it binds to the presynaptic junction of motor neurons and prevents the release of the neurotransmitter acetylcholine. This lack of signal to the particular muscle group innervated by the neuron causes a flaccid paralysis. Death can result from the toxins action on muscles of the respiratory system including the diaphragm and the intercostals.

Type A toxin when diluted to very low concentrations (ng/ml) is currently used therapeutically for the treatment of spastic muscle disorders (Jankovic and Briä, 1991; Savino and Maus, 1991). Target muscle groups are injected directly causing a regional paralysis and denervation (Borodic et al., 1991). A single batch of purified toxin was approved by the U. S. Food and Drug Administration for treatment of disorders such as strabismus, blepharospasm, and hemifacial spasm in December of 1989 (Schantz and Johnson, 1992). This particular batch was produced by the Hall A strain of C. botulinum in a simplified culture medium consisting of casein hydrolysate, yeast extract, and glucose.

Production of type A toxin by the Hall strain in a simplified growth medium was first elucidated by Lewis and Hill (1947). The purpose of their research was to establish that the organism could produce toxin titers in the range of 5×10^5 - 1×10^6 mouse lethal

doses/ml of culture in such a medium. This research was pursued due to difficulties in World War II in obtaining the necessary ingredients for the infusion broth of Wagner et al. (1925) and Dack and Wood (1928). Type A toxin production in the medium of Lewis and Hill (1947) was sufficient to enable crystallization of the toxin for the first time by Lamanna et al. (1946). A similar medium was developed by Nigg et al. (1947) for preparation of a type A toxoid in which no allergenic substances were desired.

In this study, three batches of crystalline type A toxin were purified using a modification of the method of Duff et al. (1957). The three batches were characterized at each step in the purification procedure for toxicity, % solids, absorbance values at 260 and 278 nm, and toxin yield. The proteins present after each step were analyzed by SDS-PAGE to assess purity of the toxin complex. One batch of toxin was selected for further analysis with regard to RNA content of the toxin at various steps in the purification process. Toxin production and recovery was variable with different individual peptones in the medium indicating that nutrition affected toxin quality.

Materials and Methods

Bacterial strains and culture production.

The Hall A strain of type A *Clostridium botulinum* was used to produce type A toxin complex. This strain was selected because of the high toxin titers it produces as well as the high degree of cell lysis observed during culturing. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers by Dr. E. J. Schantz and coworkers at Fort Detrick, MD. Stock cultures of *C. botulinum* Hall A were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3% dextrose (CMM; Difco Laboratories, Detroit, MI) under an anaerobic atmosphere (80% N₂, 10% CO₂, 10% H₂) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain routinely gave toxin titers in excess of 10⁶ intraperitoneal LD₅₀/ml (LD₅₀/ml) in 48 h.

Cultures for toxin purification were grown statically in either 12 or 15 liter volumes of 2.0% trypticase peptone (BBL, Cockeysville, MD), 0.75% bacto-peptone (Difco Laboratories, Detroit, MI), 1.0% yeast extract (Difco), 0.5% glucose, pH 7.4, in glass carboys of 15 or 20 liters at 37°C until such time as the culture was harvested (usually 5-7 days). Alternatively, some cultures were grown in 1-2 liter of 2.0% casein hydrolysate (Sheffield Inc., Norwich, NY), 1.0% yeast extract (Difco), 0.5% glucose, pH 7.4, in Erlenmeyer flasks. Glucose was autoclaved separately in a 50% solution and aseptically added to the remainder of the medium after cooling.

Toxin purification.

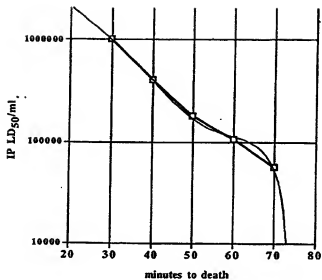
Type A toxin complex was purified in the manner approved by the U. S. Food and Drug Administration which is a modification of the method of Duff et al. (1957). The method consists of growing type A *C. botulinum* Hall A for 5-7 days in the simplified

casein hydrolysate medium at 37°C. At this time the pH of the crude culture was lowered to pH 3.4 with 3N H₂SO₄ and stirring. The toxin as well as many other proteins as well as cellular debris in the culture form large flocs and precipitate under these conditions and settle to the bottom. The precipitated material containing the toxin is termed the first acid mud. Toxin at this point is stable for years when stored at 4°C. The precipitated material was separated from the supernatant by decanting and the precipitate allowed to further settle overnight at room temperature. The precipitate was collected by centrifugation at (4,100 x g for 20 min at 5-10°C) and washed once with dH₂O. The first acid precipitate was then homogenized in dH₂O and the volume brought to 925ml. The toxin was extracted by the addition of CaCl₂ to a final concentration of 75mM (75ml of a 1.0M CaCl₂ solution) and raising the pH to 6.5-6.8 with 1N NaOH. The extraction was continuously stirred at room temperature for 2-4 h at which time the extracted toxin was separated from the cellular debris by centrifugation at 16,000 x g for 20 min at 5-10°C. The toxin was then immediately reprecipitated by lowering the pH to 3.7 with 1N HCl. This is termed the second acid precipitation and is generally allowed to settle overnight at 4°C. Precipitated material containing the toxin was collected from this second acid mud by centrifugation (16,000 x g for 20 min at 5-10°C). Toxin was reextracted with constant stirring in 150ml of 50mM sodium phosphate, pH 6.8, for 2-4 h at room temperature. The extracted toxin was separated from the insoluble material by centrifugation (16,000 x g for 20 min at 5-10°C) and the volume of the toxin extract made up to 150ml with 50mM sodium phosphate, pH 6.8. The toxin extract was then cooled to 0-2°C in a glycerol:ethanol:H₂O constant temperature bath. Alternatively, this cooling step may be done directly in a refrigerated centrifuge. When the toxin cooled to below 2-3°C, 65ml of 50% ethanol (precooled to -20°C) was slowly added with stirring over a period of 30-45 min taking care not to let the temperature rise above 2-3°C. The toxin was then cooled to -5°C and allowed to stand overnight under these conditions. Ethanol precipitated toxin was recovered by

centrifugation ($16,000 \times g$ for 20 min at -5°C) and the supernatant discarded. A small quantity (ca. 25 ml) of 50 mM sodium phosphate, pH 6.8, chilled to ca. 0°C was immediately added to dilute the residual ethanol and the pellet gently homogenized in a further volume of 50 ml of 50 mM sodium phosphate, pH 6.8. Toxin was gently dissolved in a final volume of 75 ml of 50mM sodium phosphate, pH 6.8, at room temperature for 1-2 h. The solution was clarified by centrifugation ($12,000 \times g$ for 20 min at $5-10^{\circ}\text{C}$) and the final volume made to 75 ml with 50 mM sodium phosphate, pH 6.8. The toxin was crystallized for the first time by the slow addition (15 min) of 20 ml of 4M $(\text{NH}_4)\text{SO}_4$ with continuous stirring. The toxin crystallized in 1-4 days at 4°C at which time the crystals were collected by centrifugation ($12,000 \times g$ for 20 min at $5-10^{\circ}\text{C}$). Crystalline toxin was dissolved in ca. 50 ml of 50 mM sodium phosphate, pH 6.8, clarified by centrifugation ($12,000 \times g$ for 20 min at $5-10^{\circ}\text{C}$), and the volume brought to 75 ml with 50mM sodium phosphate, pH 6.8. The toxin was crystallized a second time in the same fashion by addition of 20 ml of 4M $(\text{NH}_4)\text{SO}_4$ and incubating at 4°C . In some cases a third crystallization was performed by the same procedure.

Toxin assays.

Toxicity of various preparations were estimated using 18-22g female, ICR strain mice and the intravenous method of Boroff and Fleck (1966). The time-to-death method is converted to intraperitoneal LD_{50}/ml using the standard curve shown in Figure 1. Toxin concentrations are adjusted in order that the time-to-death values lie in the linear portion of the curve (30-70 min). In most assays three mice are injected and an average time-to-death value calculated. In some cases five mice are used and the average time-to-death calculated. Where appropriate, toxin titers were further determined using the standardized dilution to extinction method of Schantz and Kautter (1978).



$$y = -29.5x^3 + 5.33 \times 10^3 x^2 - 3.22 \times 10^5 x + 6.67 \times 10^6$$

$$r^2 = 1.000$$

Figure 1. Clostridium botulinum type A toxin (3X crystallized) time-to-death standard curve.

RNA extraction.

Whole culture samples. Samples obtained from one 15 liter batch of *C. botulinum* Hall A were taken at various time points during the incubation of the culture and at steps in the toxin purification procedure. Samples taken during the incubation of the culture had dithiothreitol (Boehringer-Mannheim Corp., Indianapolis, IN) added to a final concentration of 1mM; EDTA (Sigma Chemical Co., St. Louis, MO) added to a final concentration of 1mM, and RNase inhibitor (Boehringer-Mannheim) added at a level of 50 units/ml. [All solutions, buffers, and glassware were made in diethyl pyrocarbonate (DEPC) treated dH₂O (0.1% DEPC (Sigma) in dH₂O, incubated ≥8 h, then autoclaved) unless otherwise noted]. These samples were then frozen at -20°C until extracted. Extraction of RNA was done by adding 100 µg/ml of lysozyme (Sigma) to the thawed and mixed samples and incubating for 15 min at 37°C. Sodium dodecylsulfate (SDS) (0.5% w/v) and Proteinase K (Pro K) (100 µg/ml) (Bethesda Research Laboratories, Gaithersburg, MD) were added and the mixture incubated at 50°C for 1 h. One-tenth volume of 3 M sodium acetate (Sigma) was added and the solution transferred to DEPC-treated centrifuge tubes. The solution was extracted once with an equal volume of 65°C TBE equilibrated phenol (TBE= 45mM Tris-HCl, 45mM sodium borate, 1mM sodium ethylene diamine tetraacetate, pH 8.0. Sambrook et al., 1989) by vortexing for 10 sec and cooling on ice. The aqueous phase was transferred to new centrifuge tubes after centrifuging at 12,000 x g for 20 min at 4°C and an equal volume of isopropanol added. Tubes were stored overnight at -20°C. Faint pellets visible after centrifugation at 12,000 x g for 60 min at 4°C were dissolved in 300 µl of dH₂O containing 50 units of RNase inhibitor and 35 units of DNase I (BRL). Following incubation at room temperature for 1.5 h three volumes of 4 M ammonium acetate, pH 4.5, were added and the mixture held on ice for 1 h. The pellets recovered by centrifugation at 12,000 x g for 30 min at room temperature were dissolved in 50 µl of dH₂O and reprecipitated by adding one-tenth

volume of sodium acetate, pH 4.5, 2.5 volumes 95% ethanol, and were stored overnight at -20°C. Following a wash with 70% ethanol, the pellets were dissolved in 100 µl dH₂O and extracted once with 1:1 phenol:CHCl₃ followed by a single CHCl₃ extraction. The RNA was precipitated with one-tenth volume of sodium acetate, pH 4.5, and 2.5 volumes 95% ethanol and incubated at -20°C for 2 h. The resulting pellets were dissolved in 50 µl of 1X TBE and quantitated by absorbance at 260nm assuming an absorbance of 1.0 = a 40 µg/ml solution of RNA.

RNA extraction from toxin purification samples.

Samples taken during the purification of crystalline type A toxin were treated in a similar fashion with the following exceptions; after Pro K digestion, 65°C phenol extraction, and isopropanol precipitation, the pellet was dissolved and the aqueous phase extracted with 1:1 phenol:CHCl₃ until no interface was visible (usually 2-4 times). The aqueous phase was then extracted once with CHCl₃. Samples from the first and second crystallizations were not treated with lysozyme.

SDS-gel electrophoresis.

Electrophoresis was performed using a Pharmacia Phast System (Pharmacia LKB Biotechnology, Piscataway, NY) and 12.5% linear pre-cast gels according to the manufacturers instructions. Sample buffer consisted of 75 mM Tris-HCl (Sigma), 5 M urea (Sigma), 5% SDS (Sigma), and 20% glycerol (Sigma), pH 6.8. All samples were boiled for 5-10 min. Some samples were reduced by the addition of 0.5% dithiothreitol. Bands were visualized by staining in 0.1% Coomassie brilliant blue R250 in 40% methanol, 20% acetic acid, destained in 25% methanol, 7.5% acetic acid followed by silver staining according to the procedure of Hammes (1990).

Agarose gel electrophoresis.

RNA samples were electrophoresed in 0.9% agarose-formaldehyde gels at 80V (constant voltage) according to the procedure of Sambrook et al. (1989).

Estimation of protein concentration.

Protein concentrations of crude extracts were estimated using the method of Smith et al. (1985) with bovine serum albumin as the standard. When working with purified forms of the toxin complex, protein concentration was estimated using the extinction coefficient at 278nm of $1.65 = 1 \text{ mg/ml}$ of the toxin complex in a 1cm light path (Knox et al., 1970).

Results

Batch-to-batch characterization.

Purification of type A toxin complex using the U. S. Food and Drug Administration approved method of Duff et al. (1957) is shown schematically in Figure 2. Representative samples were taken at the various steps for analysis including optical densities, % solids, % recovery, and specific toxicity. These results are summarized in Table 1.

Production of crystalline type A toxin complex using the precipitation and crystallization method of Duff et al. (1957) appears to be a variable process (Table 1). Variation is introduced even before the seed culture is inoculated into the fermentation vessel; medium components play a very important role in the production and subsequent properties of the toxin. Using the same peptone from the same manufacturer but of a different lot had dramatic results on final yield of toxin (Table 2).

It is generally possible to recover 10-20% of the starting titer using the method of Duff et al. (1957) which compares favorably with the value of 17% originally reported by them. The final yields expected based on the starting titers and the assumption that the toxin in the crude culture had a specific activity of 30 LD₅₀/ng were: Batch 1, 13.7%; Batch 2, 11.4%; and Batch 3, 18.4%. Batch 1 had a yield of >25% of the starting toxicity after two crystallizations. However, the optical density (OD) ratio of 260/278nm was out of the accepted range of <0.6 recommended for high quality type A toxin complex for medical use (Schantz and Johnson, 1992). This necessitated a third crystallization in which another 12% of the toxin was lost. Recovery of toxin from one step to the next in the purification scheme was another source of variation. The acid precipitation steps at the outset were about 90% effective in recovering the toxin from solution. Toxin can be lost when extraction of the acid precipitates is incomplete, however, some losses are acceptable in the interest of time and simplicity. Repeated extractions of the acid mud can recover a

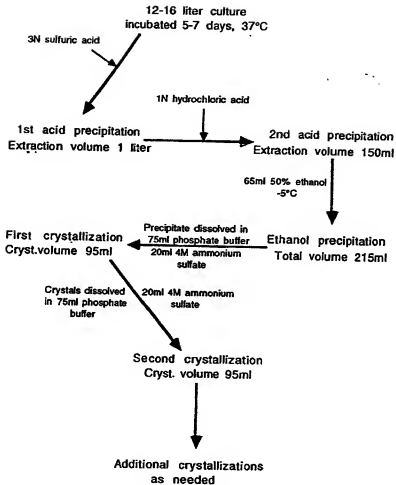


Figure 2. *Clostridium botulinum* type A crystalline toxin purification scheme.

Table 1. Batch-to-batch characterization of *Clostridium botulinum* Hall A purified according to a modification of the method of Durr et al., (1957).

| | Batch # | 5 day culture | 1st acid ppt | 2nd acid ppt | EtOH ppt sucrose | EtOH ppt pellet | 1X crystal | 2X crystal | 3X crystal |
|-------------------|---------|----------------------|--------------|--------------|------------------|-----------------|-----------------|-----------------|-----------------|
| % solids | 1 | 3.670 | 4.233 | 2.076 | 1.168 | 1.268 | 12.74 | 9.929 | 8.654 |
| | 2 | 2.731 | 2.664 | 0.879 | 0.435 | 0.557 | 10.76 | 6.831 | |
| | 3 | 2.745 | 1.612 | 0.906 | ND | 0.685 | 12.23 | 9.470 | |
| 260nm (1:20) | 1 | 2.520 | 2.864 | 2.570 | 2.560 | 0.702 | 0.305 | 0.190 | 0.076 |
| | 2 | 2.113 | 1.499 | 1.024 | 1.470 | 0.159 | 0.149 | 0.035 | |
| | 3 | 2.109 | 2.612 | 1.699 | ND | 0.258 | 0.248 | 0.071 | |
| 278nm (1:20) | 1 | 2.310 | 2.700 | 2.910 | 2.680 | 0.717 | 0.323 | 0.250 | 0.133 |
| | 2 | 1.683 | 1.109 | 0.932 | 0.913 | 0.186 | 0.171 | 0.060 | |
| | 3 | 1.727 | 2.146 | 1.071 | ND | 0.308 | 0.318 | 0.129 | |
| ratio (260/278nm) | 1 | 1.09 | 1.06 | 0.88 | 0.99 | 0.98 | 0.94 | 0.76 | 0.57 |
| | 2 | 1.26 | 1.35 | 1.10 | 1.61 | 0.86 | 0.87 | 0.58 | |
| | 3 | 1.22 | 1.22 | 1.59 | ND | 0.84 | 0.78 | 0.56 | |
| BCA (μ g/ml) | 3 | 7600 | 5540 | 2160 | 1620 | 3810 | 1580 | | |
| total mg toxin | 1 | 1100 ^a | | | | | 376 | 285 | 151 |
| | 2 | 600 ^a | | | | | 195 | 68.4 | |
| | 3 | 800 ^a | | | | | 362 | 147 | |
| toxicity | 1 | 2.2x10 ^{6b} | | | | | 12 ^c | 22 ^c | 20 ^c |
| | 2 | 1.5x10 ^{6b} | | | | | 11 ^c | 18 ^c | |
| | 3 | 2.0x10 ^{6b} | | | | | 19 ^c | 28 ^c | |

^atotal mg are estimated using 30 LD₅₀/ng of toxin complex; ^btoxicity is given in LD₅₀/ml of crude culture; ^ctoxicity is given in LD₅₀/ng of toxin complex.

Table 2. Toxin production by *Clostridium botulinum* Hall A after 7 days in various casein hydrolysates and recovery following acid precipitation.*

| Peptone ¹ | Toxin titer after 7 days ² | pH after 7 days | Titer after lowering pH to 3.4 ³ | %ppt'd ⁴ |
|----------------------|--|--------------------|--|---------------------|
| TT lot# 9NC29 | 1.36×10^6 | 5.76 | 6.0×10^4 | 95.6 |
| TT lot# 1NB05 | 1.16×10^6 | 5.89 | 1.9×10^5 | 83.6 |
| TT lot# 0NL30 | 8.8×10^5 | 5.85 | 1.8×10^5 | 79.5 |
| B lot# 0ND05A | 9.6×10^5 | 5.73 | 2.2×10^5 | 77.0 |
| B lot# 2ND20 | 1.5×10^6 | 5.53 | 6.8×10^5 | 54.7 |
| EKC lot# 1ND10A | 1.6×10^6 | 5.53 | $<4 \times 10^4$ | >97 |
| EKC lot# 0NK19 | 1.16×10^6 | 5.88 | 2.1×10^5 | 82.1 |
| EKC lot# 1NK18 | 6.4×10^5 | 5.87 | $<8 \times 10^3$ | >95 |
| A lot# 1ND01V | 9.6×10^5 | 5.78 | 1.1×10^5 | 88.5 |

*Medium consisted of 1 liter of 2.0% casein hydrolysate, 1.0% yeast extract (Difco), 0.5% glucose, pH 7.4.

¹All peptones were obtained from Sheffield Laboratories, Inc., Norwich, NY.

²Toxicity was estimated using the intravenous method of Boroff and Fleck (1966).

³Toxicity of supernatants was determined after acidification with 3N HCl to pH 3.4 and allowing precipitate to settle for 24 h.

⁴% precipitated = starting titer - supernatant titer / starting titer X 100.

higher percentage of the toxin (data not shown). Crystallization of the type A toxin complex results in losses due to the fact that not all of the toxin present associates into crystalline form (Sugiyama et al., 1977). A scanning electron micrograph of crystalline type A toxin complex is shown in Figure 3. Losses of toxin from one crystallization to the next are generally on the order of one-third to one-half of the total. In this study losses ranged from 32-65% of the total amount of toxin present from one crystallization to the next. The benefit gained from additional crystallizations is usually a decrease in the absorbance ratio (260/278nm) and generally an increase in specific toxicity. However, decreases in specific activity are not uncommon presumably due to increased handling of the toxin.

Specific activity of the final product is extremely important in pharmaceutical formulations. The higher the specific activity the fewer the number of nanograms required in a given vial since each vial (for sale in the U. S.) contains 100 LD₅₀. Decreasing the quantity of toxin injected is desirable since there are currently patients who are producing neutralizing antibodies to the toxin (Jankovic and Schwartz, 1991). From Table 1 it can be seen that final specific toxicities were variable. Average specific toxicities for these toxin batches as well as subsequent batches not described were in the range of 18-28 LD₅₀/ng. These toxicities are somewhat lower than those which can be routinely obtained using chromatographic purification methods, in which the specific activity of the toxin is usually in excess of 30 LD₅₀/ng of toxin complex (Sugiyama et al., 1977).

SDS-PAGE analysis of toxin samples.

Samples taken at the various stages of type A toxin purification shown in Figure 2 were examined using reducing and non-reducing sodium-dodecylsulfate gel electrophoresis. The results are shown in Figures 4-7. The final crystallizations of the three batches are compared side-by-side in Figure 8.



Figure 3. Scanning electron micrograph of crystalline type A toxin magnified 10,000X. The crystal was ca. 6 μm in length.

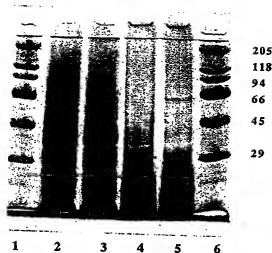


Figure 4. SDS-PAGE of whole culture and first acid precipitation samples taken during purification of *C. botulinum* type A toxin complex, batch 2. Lanes 1 and 6, molecular weight markers (rabbit myosin- 205 kDa, β -galactosidase- 118kDa, *E. coli* phosphorylase b- 94 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, and bovine erythrocyte carbonic anhydrase- 29 kDa) (Sigma), 4-5 μ g protein total. Lane 2, 5 day whole culture (unreduced); lane 3, 5 day whole culture (reduced with 0.5% w/v dithiothreitol); lane 4, extract of first acid precipitate (unreduced); lane 5, extract of first acid precipitate (reduced), 4-6 μ g protein each lane.

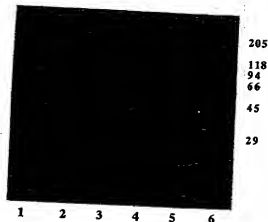


Figure 5. SDS-PAGE of extract from second acid precipitation and supernatant from ethanol precipitation taken during purification of *C. botulinum* type A toxin complex, batch 2. Lanes 1 and 6, molecular weight markers (rabbit myosin- 205 kDa, β -galactosidase- 118kDa, *E. coli* phosphorylase b- 94 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, and bovine erythrocyte carbonic anhydrase- 29 kDa) (Sigma), 4-5 μ g protein total. Lane 2, extract of second acid precipitate (unreduced); lane 3, extract of second acid precipitate (reduced with 0.5% w/v dithiothreitol); lane 4, supernatant from ethanol precipitation (unreduced); lane 5, supernatant from ethanol precipitation (reduced), 4-6 μ g protein each lane.

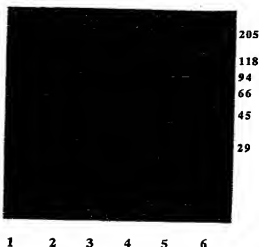


Figure 6. SDS-PAGE of pellet from ethanol precipitation and first crystallization taken during purification of *C. botulinum* type A toxin complex, batch 2. Lanes 1 and 6, molecular weight markers (rabbit myosin- 205 kDa, β -galactosidase- 118kDa, *E. coli* phosphorylase b- 94 kDa, bovine serum albumin- 66 kDa, ovalbumin- 45 kDa, and bovine erythrocyte carbonic anhydrase- 29 kDa) (Sigma), 4-5 μ g protein total. Lane 2, pellet from ethanol precipitation (unreduced); lane 3, extract from pellet of ethanol precipitation (reduced with 0.5% w/v dithiothreitol); lane 4, 1X crystallized toxin complex (unreduced); lane 5, 1X crystallized toxin complex (reduced), 4-6 μ g protein each lane.

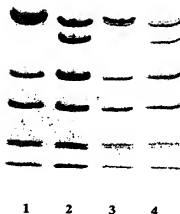


Figure 7. SDS-PAGE of 2X crystallized toxin complex taken during purification of *C. botulinum* type A toxin complex, batch 2 compared to type A toxin complex purified by the chromatographic method of Tse et al. (1982). Lane 1, 2X crystallized type A toxin complex (unreduced); lane 2, 2X crystallized type A toxin complex (reduced with 0.5% w/v dithiothreitol); lane 3, type A toxin complex purified by the method of Tse et al. (1982) (unreduced); lane 4, type A toxin complex purified by the method of Tse et al. (1982) (reduced), 4-6 μ g protein each lane.



Figure 8. SDS-PAGE of toxin batches 1 (lanes 1, 2), 2 (lanes 3, 4), and 3 (lanes 5, 6). Samples in odd numbered lanes are unreduced, samples in even numbered lanes were reduced with 0.5% dithiothreitol.

Medium component dependence of toxin production.

The production of botulinum toxin was dependent on the medium used. The component of the growth medium which appeared to have the greatest bearing on final toxicity and recovery by acid precipitation was casein hydrolysate. Some of the differences observed in the batches of toxin shown (Table 1) were probably caused by the use of different lots of trypticase peptone. To further assess the role of casein hydrolysate on toxin recovery, 4 different types of casein hydrolysates comprising 9 different lots were obtained. Each casein hydrolysate was used in a medium consisting of 2.0% casein hydrolysate, 1.0% yeast extract, and 0.5% glucose, pH 7.4, in a final volume of 1-2 liters. These were inoculated with 0.25 ml of the same 24 h old culture of *C. botulinum* Hall A. Incubation was carried out statically for 7 days at which time the cultures were assayed for toxicity and acidified with 3 N HCl (Table 2). The range of final toxicities between different lots of the same peptone varied from 8.8×10^5 - 1.36×10^6 LD₅₀/ml for NZ amine TT, 6.4×10^5 - 1.6×10^6 LD₅₀/ml for NZ amine EKC, and 9.6×10^5 - 1.5×10^6 LD₅₀/ml for NZ amine B. The only sample of NZ amine A used gave a final toxin titer of 9.6×10^5 LD₅₀/ml. High toxin titers were not attributable to final pH values following culture as hydrolysates which produced toxin titers in excess of 10^6 LD₅₀/ml had final pH values from 5.53-5.89 while those producing less than 10^6 LD₅₀/ml had final pH values of 5.73-5.87.

In addition to high toxin titer, another important criterion for peptones intended for toxin production is recovery of the toxin during the first step of purification. When the pH is lowered to 3.4 to precipitate the toxin, the toxin should form large flocs and settle rapidly (<1 h after final acid addition). The casein hydrolysates varied in the degree to which the toxin precipitated after culturing (Table 2). For example, NZ amine B lot# 2ND20 gave the highest toxin titer among the hydrolysates tested but also gave the lowest recovery of toxin following acidification. The opposite was also observed: NZ amine EKC lot#

INK18 gave more than 95% recovery but initially produced only 40% as much toxin as produced in the NZ amine B lot# 2ND20. These results clearly show that casein hydrolysate markedly affected production and recovery of toxin and that more work is necessary to further elucidate the factors involved in toxin production.

RNA content of toxin.

RNA is well known to be associated with type A botulinum toxin complex (Tse et al., 1982; Schantz and Johnson, 1992), and we were interested in determining whether specific classes of RNA were bound to the complex and carried through the protein purification. RNA was extracted from cultures of *C. botulinum* Hall A at different time points during incubation (Table 3). The extraction procedure recovered RNA which was analyzed on a 0.9% agarose-formaldehyde gel according to the procedure of Sambrook et al. (1989). The analysis showed 2-3 prominent bands presumably representative of ribosomal RNA (Figure 9, lanes 3-6). As the culture aged, the quantity of lower molecular weight species increased (Figure 9, lanes 5, 6). As the culture grew from early exponential phase to stationary phase (8 h-24 h), the amount of RNA also increased (Table 3). As the cells began to lyse at around 24-48 h, the amount of RNA dropped from a maximum of 106.6 μg RNA/ml of culture to a low of 10.6 μg /ml. RNA degrading enzymes are abundant in bacteria and the concentration of RNA in the culture medium should decrease following lysis.

One batch of toxin (batch 3) was assayed at various points in the purification procedure for RNA which might be associated with the toxin complex (Table 4). The amount of RNA associated with the toxin complex dropped during the purification. Approximately 3.4 μg of RNA/mg of toxin complex was detected in the 2X crystallized material or ca. 0.3% RNA/mg toxin. RNA was present as low molecular weight species

and none of the fragments were larger than ca. 800 bases with most less than 300 bases (Figure 10, lanes 1-7).

Table 3. Quantities of RNA extracted from Clostridium botulinum Hall A whole culture after various incubation times.

| <u>Sample</u> | <u>µg RNA/ml culture</u> |
|---------------|--------------------------|
| 8 h | 49.6 |
| 24 h | 106.6 |
| 48 h | 25.5 |
| 120 h | 10.6 |

Table 4. Quantities of RNA extracted from toxin preparations at various steps in purification of crystalline type A toxin, batch 3.

| Sample | $\mu\text{g RNA/}$ ml sample | mg protein/ ml sample^1 | $\mu\text{g RNA/}$ mg protein | ratio (260/278) |
|---------------|--|--|---|--------------------|
| 2nd acid ppt | 576 | 2.10 | 274 | 1.85 |
| EtOH ppt/supr | 292 | 1.62 | 180 | 1.85 |
| EtOH ppt/pell | 169 | 3.83 | 44 | 1.86 |
| 1X cryst/supr | 44 | 3.88 | 11.3 | 1.90 |
| 1X cryst/pell | 18.8 | 3.91 | 4.8 | 1.89 |
| 2X cryst | 8 | 2.37 | 3.4 ² | 1.88 |

¹based on BCA assay data of total protein from batch 3, Table 1.

²batch 3 had a 260/278nm absorbance ratio of 0.56 and a specific toxicity of 28 LD₅₀/μg.



Figure 9. 0.9% agarose-formaldehyde gel of RNA from whole culture of *Clostridium botulinum* type A Hall strain. Lane 1, Hind III digestion of lambda DNA, (23 kb, 9.4 kb, 6.6 kb, 2.3 kb, 2.0 kb, 0.56 kb); lane 2, blank, lane 3, RNA extract from 8 h culture of Hall A, lane 4, RNA extract from 24 h culture of Hall A, lane 5, RNA extract from 48 h culture of Hall A, lane 6, RNA extract from 120 h culture of Hall A, ca. 1 μ g total each lane.

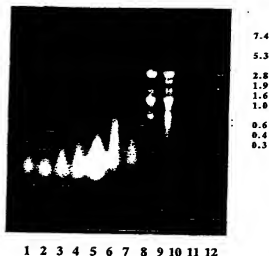


Figure 10. 0.9% agarose-formaldehyde gel of RNA from purification of toxin complex of *Clostridium botulinum* type A (Hall strain), batch 3. Lane 1, second acid precipitate; lane 2, ethanol precipitate (supernatant); lane 3, ethanol precipitate (pellet); lane 4, first crystallization (supernatant); lane 5, first crystallization (crystalline toxin); lane 6, second crystallization (supernatant); lane 7, second crystallization (crystalline toxin); lane 8, RNA extraction from 24 h culture Hall A; lane 9, RNA extraction from 120 h culture Hall A. Lanes 1-9 were each loaded with ca. 2-3 μ g RNA extract each. Lane 10, blank; lane 11, BioRad low molecular weight RNA markers (1.6 kb, 1.0 kb, 0.6 kb, 0.4 kb, 0.3 kb), ca. 1 μ g total; lane 12, BioRad high molecular weight RNA markers (7.4 kb, 5.3 kb, 2.8 kb, 1.9 kb, 1.6 kb), ca. 1 μ g total.

Discussion

The purification of type A botulinum toxin for medical use has been done by a series of precipitations and crystallizations originally elucidated in 1957 by Duff and coworkers. The precipitation method was used to produce the only batch of toxin licensed so far in the United States by the Food and Drug Administration and was made by Dr. E. J. Schantz at the Food Research Institute in 1979 (Schantz and Johnson, 1992). This particular method was chosen in part because there would be no danger of other materials eluting from columns and contaminating the final product and possibly causing allergic reactions (Schantz and Johnson, 1992). Currently, it is possible to avoid such difficulties with careful attention to the column matrix, preparation of the column, and running conditions. Also, since stock suspensions of crystalline type A toxin can easily be kept at concentrations as high as 10-20 mg/ml, diluting the toxin to therapeutic doses (ca. 10^6 X) would tend to dilute most contaminants to non-reactive levels. There are more efficient methods of purifying protein available today including various types of column chromatography (Tse et al., 1982; Woody and DasGupta, 1988), and these methods should be considered for isolating toxin with high specific toxicity.

Different peptones (casein hydrolysates) were found in this study to affect production of type A toxin complex by the Hall A strain. The peptones were incorporated at 2.0% into a solution containing 1.0% yeast extract and 0.5% glucose, pH 7.4. There were significant differences between the various kinds of peptones as well as among lots of the same kind of peptone (Table 2). The mechanisms by which peptones affect toxin production and quality are not known. From the work done with *C. tetani* in the 1950's and 60's (Mueller and Miller, 1956; Latham et al., 1962), it is possible that a given peptone contains either a limiting peptide which limits the availability of an amino acid or that the peptone contains something which is inhibiting toxin formation. Recent work with

minimal media for *C. botulinum* has elucidated some of the requirements for toxin synthesis in group I (proteolytic) and group II (non-proteolytic) strains (Leyer and Johnson, 1990; Patterson-Curtis and Johnson, 1989; Whitner and Johnson, 1988). For Group I *C. botulinum*, it was found that arginine and phenylalanine were essential for growth and toxin production. However, it was subsequently determined that high levels of arginine inhibited protease and toxin formation in both *C. botulinum* Hall A and Okra B (Patterson-Curtis and Johnson, 1989). High levels of tryptophan were shown to inhibit the production of type E botulinum toxin (Leyer and Johnson, 1990). Future work into the nutritional requirements of *C. botulinum* toxin formation may elucidate the reasons for the discrepancies observed between the various peptones.

Careful handling of *C. botulinum* during purification is critical for maximum recovery. The toxin is very susceptible to denaturation on the surface of air bubbles or sidewalls of the vessel due to agitation and in some instances temperature. There is usually some loss at each step in the purification due to these factors regardless of the purification method used. Maximizing the amount of toxin recovered is dependent mainly on the quantity produced by *C. botulinum* and the amount recovered in the first acid precipitation. The quantity of toxin produced is dependent on the lot of peptone used in the medium. The peptone composition also affects the flocculation and settling of toxin on acidification of the culture. One way around this problem of toxin staying suspended in the spent culture medium is centrifugation of the entire culture which is trivial if equipment such as continuous flow centrifuges are available but is very tedious and labor intensive if only standard centrifuges are present as cultures for bulk toxin production are typically 32-40 liters in size. Duff et al. (1957) reported that toxin also precipitated poorly when stock cultures of *C. botulinum* Hall A which had been stored at 4°C for an extended period of time and were subsequently used for toxin production. Cultures stored frozen at -20°C did not show this phenomenon suggesting that there was physiological modification of the

organism during refrigerated storage. The recovery of toxin on acidification appears to be related to the properties of the seed culture and also to the peptone composition.

When type A toxin complex is purified using the method of Duff et al. (1957), it is possible to maximize recovery at a few key steps. The rate of acid addition in both of the acid precipitations, the first to the crude culture, and the second to the extract of the first acid precipitate, does not seem to have any effect on either yield or the specific activity of the material recovered. Performing a second extraction of the first acid mud results in recovery of ca. 10-20% more toxin being recovered (data not shown). However, the 80-90% recovered in the first extraction is usually acceptable. The same results can be observed for the phosphate extraction of the second acid mud. The recovery at this step is lower (on the order of 60-70%). Subsequent reextraction of the pelleted material can recover an additional 10-15% but is usually not done.

Ethanol precipitation of the toxin is an extremely sensitive step in the purification. Toxin is denatured by ethanol at temperatures above ca. 2-3°C (England and Seifter, 1990). Great care must be taken to ensure that the temperature stays low during the exothermic addition of the 50% ethanol solution and that mixing is rapid. Recently, we found that doubling the volume of the ethanol precipitation from 215 ml of chilled toxin extract to 430 ml for large (>30 liter of crude culture) batches of toxin greatly increased the percent yield at this step. Two possible explanations for the ca. 5-8 fold increase in toxin recovered are that the ethanol as a precipitating agent was somehow limiting on the smaller scale or more likely that the larger volume of chilled toxin resists temperature fluctuation due to ethanol addition keeping the temperature low. Following the overnight -5°C incubation, the precipitated toxin is centrifuged from the ethanol solution and the ethanol decanted as rapidly as possible. Here, doing the ethanol precipitation step in the centrifuge itself means that the toxin does not have to be transferred from the precipitation vessel to a centrifuge bottle allowing the toxin to warm slightly. After the centrifugation step, the supernatant is

decanted and the pellet immediately covered with 0°C 50mM sodium phosphate buffer, pH 6.8, and homogenized with a glass rod. This step must be done quickly to ensure that residual ethanol present with the pellet is diluted to minimize denaturation of the toxin as the temperature rises. Toxin is unavoidably lost at this point due to surface denaturation from the mechanical stirring.

Crystallization of the toxin complex is accomplished by the addition of 4M ammonium sulfate to a final concentration of ca. 0.8-0.9M. This amount of ammonium sulfate is lower than that which causes precipitation (ca. >50% saturated ammonium sulfate or 2.0 M). Presumably, the ammonium sulfate acts to reduce the ionic interactions between the toxin complex molecules to the point which favors protein-protein interactions necessary for alignment into a crystalline structure between individual toxin complex molecules. It may be that without ammonium sulfate present the ionic interactions are random causing arrangements unsuited to crystallization but with low concentrations of the salt present only interactions that are mediated by weaker, shorter range forces such as hydrophobic interaction and van der Waals forces are present which may allow proper alignment into a crystalline lattice.

The loss of toxin from one crystallization to the next is substantial (on the order of one-third to one-half). In order to minimize the number of crystallizations needed to lower the 260/278 nm absorbance ratio to the required 0.5-0.6 range and thereby reduce overall toxin loss, the crystallizations must be done slowly and allowed to go to completion. Addition of the 20 ml of 4 M ammonium sulfate to bring the solution to a final 0.84 M concentration should be dropwise with continuous stirring. The addition should take place over a period of ca. 30 min to ensure that the toxin is not precipitated due to ammonium sulfate concentration gradients forming. Incubation times of each crystallization are variable. The crystals usually begin to form overnight at 4°C and are visible as a clouding of the originally clear to slightly straw colored solution. The crystallization continues until

the crystals themselves precipitate usually within 7-14 days. Sugiyama et al. (1977) reported that complete crystallization was achieved in several months. After 7-14 days, the crystals (Figure 3) are separated from the mother liquor by centrifugation. The crystals are dissolved in 50mM sodium phosphate buffer, pH 6.8, and the absorbance ratio at 260/278nm determined. Toxin complex present in mother liquor which has been separated from the original crystals will continue to form crystals even after >14 days incubation in the first crystallization although the process is slow. This indicates that the crystallization process is protein concentration dependent with the majority of the crystals forming relatively quickly and falling out of solution. As the concentration of toxin complex in solution decreases, the rate at which crystals form also decreases.

Another explanation for the relatively poor recovery of toxin during crystallization using the modified method of Duff et al. (1957) was provided by Sugiyama et al. (1977). They propose that toxin complex which had been chromatographically purified prior to crystallization had a higher percentage recovery possibly due to lower interference from trace amounts of contaminating proteins. Recovery following crystallization of type A toxin complex was significantly higher (80% versus 42%) in their study when the toxic material was chromatographically purified instead of ethanol precipitated.

The two-fold goal in examination of RNA extracted from samples taken during purification was to determine the amount of RNA present in the purified toxin and to examine the RNA present to see if one species of RNA, perhaps an mRNA corresponding to that of the toxin gene was present in crystalline toxin. It can be imagined that such an mRNA could bind to the toxin complex and be carried through the purification procedure. RNA extracted from samples taken during toxin purification was compared to that obtained from 24 h and 120 h whole cultures of *C. botulinum* Hall A (Figure 9). Samples from whole cultures taken at 24 and 120 h showed large molecular weight bands of ca. 2.9 kilobases (kb), 1.5 kb, and 0.8 kb, along with smaller fragments of < 0.6 kb (Figure 9,

lanes 9, 10). These bands are the approximate molecular weights indicative of 23S and 16S rRNA. In contrast, samples taken during purification of toxin complex (Figure 9, lanes 2-8) showed mainly smears of <0.6 kb which are indicative of breakdown fragments of larger RNA molecules. The RNA purified from both the whole culture samples and the samples taken during toxin purification was probed in a Northern blot using a DNA probe to the toxin gene. The probe bound non-specifically to molecular weight markers and slightly to the 2.9 kb and 1.5 kb bands of the 24 h and 120 h whole culture samples but not to the samples taken during protein purification. This result indicates that if the RNA associated with the toxin complex is a specific messenger it is not representative of toxin mRNA or that it is present in very low amounts. The RNA that is associated with the complex is most likely rRNA that has been fragmented and associates non-specifically with the toxin complex in the culture.

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CHAPTER V**Stabilization and recovery of type A and B Clostridium botulinum
neurotoxins following lyophilization**

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Abstract

Botulinum toxin is used in very small quantities medically for the treatment of spastic muscle disorders due to its ability to inhibit motor-neuron signals to muscle fibers causing a flaccid paralysis. Botulinum toxin must be lyophilized or freeze-dried to allow for shipping and handling of the relatively delicate protein. Recovery of type A and B toxin activity following lyophilization was dependent on a number of factors. Conditions were found that gave >90% recovery of the toxicity following lyophilization of solutions containing 20-2,000 mouse 50% lethal doses. Recovery of toxicity following lyophilization of type A and B toxin complex as well as the purified ca. 150 kDa toxin molecules was obtained on drying 0.1 ml when the pH was maintained below 7.0 and serum albumins or other protein excipients were used as stabilizers in the absence of sodium chloride. Temperature stability of lyophilized type A toxin preparations was improved by addition of trehalose to the serum albumin system but not by addition of sucrose or maltotriose.

Introduction

Clostridium botulinum is a heterogeneous group of gram- positive, anaerobic, endospore forming, rod-shaped organisms that have in common the characteristic that they produce one of the most potent proteinaceous neurotoxins known. The endospores of C. botulinum are found worldwide in soils, marine, and freshwater environments. C. botulinum is classified phenotypically and by the serotype of toxin produced. There are seven serotypes of toxin currently recognized (A, B, C₁, D, E, F, and G) (Simpson, 1981). The individual toxins synthesized by a given serotype are found present along with non-toxic proteins some of which have hemagglutinating properties (Sugiyama, 1980; Sakaguchi, 1983; Schantz and Johnson, 1992). There is some antigenic cross-reactivity between the non-toxic proteins. This indicates that there is some amino acid sequence homologies forming common three dimensional epitopes among the non-toxic proteins of the complexes (Somers and DasGupta, 1991).

Neurotoxins produced by C. botulinum are large molecular weight proteins of ca. 150 kDa (Sugiyama, 1980). Type A botulin toxin is initially produced as a single peptide chain or protoxin of 1295 amino acids in length (Binz, et al., 1990; Thompson et al., 1990). This protoxin must undergo post-translational proteolytic cleavage or nicking to achieve its characteristically high toxicity (Sugiyama et al., 1973). The nicking event occurs about one-third of the distance from the N-terminus. This nicking event generates the dichain molecule comprised of the 50 kDa light chain and 100 kDa heavy chain. The individual chains are connected by one disulfide bridge. The neurotoxin molecule exerts its characteristic muscle paralysis when the C-terminus region of the heavy chain binds to a receptor on motor-neuron end plates. The light chain is internalized through a channel formed by the N-terminus half of the bound heavy chain, and causes an inhibition of neurotransmitter release by a mechanism involving proteolytic cleavage of vesicle

associated membrane proteins (VAMPs) (Schiavo et al., 1993). This lack of neurotransmitter signal across the synaptic junction to the muscle causes the flaccid paralysis seen in cases of botulism (Simpson, 1989).

Type A neurotoxin produced by *C. botulinum* is present as part of a complex of at least seven different noncovalently bound proteins (Somers and DasGupta, 1990). In culture media this toxin complex associates into dimers or trimers with a molecular weight of about 600 and 900 kDa, respectively (Tse et al., 1982; Habermann and Dreyer, 1986). The type A neurotoxin molecule has a molecular weight of 145 kDa (Gimenez and DasGupta, 1993) and in its fully active state consists of two separate peptide chains of 93 and 52 kDa that are connected by a disulfide link between cysteine residues 430 and 454 (Binz et al., 1990; Thompson et al., 1990). Type B toxin produced in culture is a mixture of two different size complexes. The larger or L complex has a molecular weight of ca. 500 kDa while the smaller or M complex is ca. 300-350 kDa (Kozaki et al., 1974; Sakaguchi, 1983). In contrast to the type A Hall strain, proteolytic type B strains do not fully activate the neurotoxic protoxin molecule associated with both complexes consequently a significant proportion of the toxin in the complex is present in the protoxin form. In these studies the two different size type B toxin complexes were not separated.

After some 23 years of development, a single batch of type A crystalline toxin complex was licensed by the Food and Drug Administration for medical use in the United States (Schantz and Johnson, 1992). This batch (#79-11) was produced at the University of Wisconsin-Madison, Food Research Institute in 1979 by Dr. E. J. Schantz, and is currently used in the treatment of hyperactive muscle disorders and dystonias due to its mode of action (Borodic, 1991; Jankovic, 1991; Scott, 1989). Disorders approved for treatment in the United States include blepharospasm, strabismus, and hemifacial spasm. Other dystonias being treated with type A toxin complex on an investigational basis worldwide include torticollis, aberrant regeneration of the seventh facial nerve, myofascial

pain syndromes, and others (Borodic, 1991). Treatment of patients involves injecting very small quantities (nanograms) of the toxin directly into affected muscle groups causing a regional decrease in muscle hyperactivity. High quality type A toxin complex for medical use has a specific toxicity of 3×10^7 mouse intraperitoneal 50% lethal doses per mg (LD_{50}) (Schantz and Johnson, 1992).

Botulinal toxin is very susceptible to denaturation due to surface denaturation, heat, and alkaline conditions. Lyophilization of botulinal toxin is the most economically sound and practical method of distributing the product in a form that is stable and readily used by the clinician. The current commercial type A botulinal toxin product is made by combining up to 500 ng/ml of type A toxin complex in 5.0 mg/ml human serum albumin (HSA) with 9.0 mg/ml sodium chloride at a pH of 7.3. After dissolution, 0.1 ml is dried to obtain 100 ± 30 active U of toxin, 0.5 mg of HSA, and 0.9 mg of sodium chloride per vial. This product has a saline concentration of 0.9 % when reconstituted in 1.0 ml of dH_2O . The current formulation gives considerable loss (up to 90 %) of activity during drying (Goodnough and Johnson, 1992) causing formation of inactive toxin that probably serves as a toxoid inciting antibody formation. In large dose applications, antibodies have been detected in patients that have become refractory to treatment (Greene, 1987; Borodic, 1991; Jankovic, 1991).

One goal of our research has been to improve recovery of active toxin following lyophilization. This reduces the amount of toxin required to obtain 100 active LD_{50} per vial. This improvement would also reduce the amount of inactive toxin in each vial and would lessen the possibility of antibody formation after injection of the preparation into patients. We have developed formulas which allow high recovery (>90%) of both type A and type B toxin complexes as well as purified type A and B neurotoxins.

The current commercial product must be stored at a temperature of $-10^\circ C$ or less to maintain the labelled potency for the one year shelf life. The product would be

considerably improved if conditions were developed that maintained shelf-stability at higher storage temperatures. This would facilitate more practical shipping and storage of the toxin. In this study, we have improved the freeze-drying formula of Goodnough and Johnson (1992) by adding carbohydrate excipients (chiefly trehalose) to increase the glass transition temperature of the dried material and thereby increasing the usable storage temperature. This improvement should enhance the temperature stability and lessen the risk of loss in potency with corresponding degradation and increase in antigenic potential.

Materials and Methods

Bacterial strains and culturing.

The Hall A strain of type A *C. botulinum* was used to produce crystalline type A complex. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers at Fort Detrick, MD by Dr. E. J. Schantz and coworkers. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 h). Type B toxin was produced from the proteolytic Okra B strain of *C. botulinum*. This strain was obtained from the Food Research Institute culture collection.

Stock cultures of *C. botulinum* Hall A and Okra B were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3 % dextrose (CMM, [Difco Laboratories, Detroit, MI]) under an anaerobic atmosphere (80% N₂, 10%CO₂, 10%H₂) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain give toxin titers in excess of 10⁶ LD₅₀/ml in 48-72 h. CMM cultures of the Okra B strain give toxin titers in the range of 5-9 x 10⁵ LD₅₀/ml in 48-96 h. Type B toxin titers may be increased by trypsinization to increase the proportion of fully active nicked toxin molecules (DasGupta and Sugiyama, 1977). However, this procedure would necessitate the removal of trypsin and was not done in this study.

For toxin production, cultures of Hall A and Okra B were grown statically in 12-15 liter volumes of toxin production medium (TPM) consisting of 2.0% NZ TT (lot # 9NC29) casein hydrolysate (Sheffield Laboratories, Norwich, NY), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37°C. Cultures of Hall A and Okra B showed heavy growth in this medium during the first 24-48 h followed by autolysis of the culture which was evident as a clearing and settling over the next 48-120 h. The Okra B strain did not produce toxin titers as high as Hall A nor did it lyse as rapidly or completely.

Type A toxin complex purification.

Type A toxin complex for use in drying studies was purified from culture broth using both the FDA-approved method involving precipitation and crystallization of the toxin complex (a modification of the method of Duff et al., 1957; see Chapter IV, this thesis) and by a method using preparative column chromatography (a modification of the method of Tse, et al., 1982). To prepare crystalline type A toxin, the 5-7 day culture was acidified with 3 N sulfuric acid to pH 3.4 and the resulting precipitate containing the toxin complex collected and extracted by adding CaCl_2 to a final concentration of 75 mM and raising the pH to 6.5-6.8. The extract was stirred for 2-4 h at room temperature and the toxin, now in solution, separated from the cellular debris by centrifugation (12,000 x g, 5-10°C, 20 min). The extracted toxin was reprecipitated by the addition of 1N HCl to a final pH of 3.7 and collected by centrifugation (12,000 x g, 5-10°C, 20 min). The toxin pellet was dissolved in 50 mM sodium phosphate buffer, pH 6.8, clarified by centrifugation (12,000 x g, 5-10°C, 20 min) and the volume made to 150 ml with buffer. Extracted toxin was cooled to 0-2°C in a constant temperature bath and 65 ml of 50 % ethanol precooled to -20°C added (15% final concentration) slowly with stirring over 30 min. The ethanol precipitate was incubated overnight at -2 to -5°C. The toxin was collected by centrifugation in a refrigerated centrifuge (12,000 x g, -5°C, 20 min) and the supernatant rapidly decanted. Residual ethanol was diluted by addition of 25-50 ml of 0°C 50 mM sodium phosphate buffer, pH 6.8, and the pellet gently homogenized with a glass rod. The toxin was dissolved to a final volume of 75 ml of the phosphate buffer, clarified by centrifugation (12,000 x g, 5-10°C, 20 min) and crystallized by the addition of 20 ml of 4 M ammonium sulfate (ca. 0.9 M final concentration). Toxin crystals were allowed to form at 4°C for 7-14 days. Crystals were collected by centrifugation (12,000 x g, 5-10°C, 20 min), redissolved in 75 ml of 50 mM sodium phosphate buffer, pH 6.8, and recrystallized by adding 20 ml of 4 M ammonium sulfate. The type A crystalline toxin used for these

studies was crystallized twice in the presence of 0.9 M ammonium sulfate and had a 260/278 nm absorbance ratio of 0.53. The extinction coefficient for type A toxin complex is $A_{278} 1.65 = 1 \text{ mg/ml}$ (Knox et al., 1970).

Type A toxin complex was also purified chromatographically by the method of Tse et al. (1982). Extracts of the first acid precipitated material were dialyzed against 50 mM sodium citrate, pH 5.5, and chromatographed at room temperature on a 1 liter DEAE-Sephadex A-50 column (Sigma Chemical Co., St. Louis, MO) equilibrated with the same buffer. One-tenth the column volume or less was chromatographed in a single passage with the toxin complex eluting in the first column volume without a gradient. Fractions from this protein peak which had a 260/278 absorbance ratio of less than 0.6 were pooled and precipitated by the addition of solid ammonium sulfate to ca. 60% saturation (39 g/100 ml).

Type A neurotoxin purification.

Type A neurotoxin was purified from the associated non-toxic proteins of the complex by a modification of the method of Tse et al. (1982). Toxin complex recovered from the DEAE-Sephadex A50, pH 5.5 column was precipitated by addition of 39 g of solid ammonium sulfate/100ml. The precipitated toxin complex was collected by centrifugation (12,000 x g, 5-10°C, 20 min), dialyzed against 25 mM sodium phosphate, pH 7.9, and applied to a DEAE-Sephadex A50 column equilibrated with the same buffer. The binding capacity of this particular matrix under these conditions is 0.9 mg of complex/ml of swollen gel. Various size columns were utilized by applying ca. 90% of the column binding capacity. Toxin was separated from the non-toxic proteins of the complex and eluted from the column with a linear 0-0.5M sodium chloride gradient. Toxin eluted from the column in the first peak and fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by adding 39 g ammonium sulfate/100 ml. Material

recovered from the DEAE-Sephadex A50 column at pH 7.9 was further purified by chromatography on SP-Sephadex C50 at pH 7.0. Precipitated toxin from DEAE-Sephadex A50 columns at pH 7.9 was collected by centrifugation ($12,000 \times g$, $5-10^{\circ}\text{C}$, 20 min) and dialyzed against 25 mM sodium phosphate, pH 7.0. The dialyzed toxin was applied to 25 ml SP-Sephadex C50 in 25 mM sodium phosphate, pH 7.0. Contaminating material did not bind to the column under these conditions. The toxin was eluted with a linear 0-0.25 M sodium chloride gradient.

Type B toxin complex purification.

Type B toxin complex was purified from crude culture fluid by a method involving the chromatographic procedure of Tse et al. (1982). Cultures of *C. botulinum* Okra B were acid precipitated to pH 3.4 using 3 N sulfuric acid. The acid mud was extracted once with 75 mM CaCl_2 and raising the pH to 6.5-6.8 analogous to purification of crystalline type A toxin. The clarified extracts were reprecipitated by lowering the pH to 3.7 with 1 N HCL. The second acid mud was then extracted with 50 mM sodium citrate buffer, pH 5.5, and the clarified extract dialyzed against the same buffer. A mixture of L and M type B toxin complexes were isolated by chromatography on 1 liter volumes of DEAE-Sephadex A50 equilibrated with 50 mM sodium citrate, pH 5.5. One-tenth the column volume or less was purified in a single passage with type B toxin complex eluting in the first column volume without a gradient. Fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by addition of 39 g ammonium sulfate/100 ml. The extinction coefficient used for type B toxin complex was $A_{278} 1.85 = 1 \text{ mg/ml}$ (Beers and Reich, 1969). This pool represented type B toxin complex with a specific toxicity of $4.2 \times 10^7 \text{ LD}_{50}/\text{mg}$.

Type B neurotoxin purification.

Type B neurotoxin was purified from the complex by a combined method involving the methods of Tse et al. (1982) and Moberg and Sugiyama (1978). Type B toxin complex in 25 mM sodium phosphate, pH 7.9, was applied to DEAE-Sephadex A50 (Sigma) equilibrated with the same buffer. Partially purified type B neurotoxin was eluted from this column with a 0-0.5 M sodium chloride gradient. Type B toxin fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by the addition of 39 g ammonium sulfate/100 ml. Precipitated material was dialyzed against 25 mM sodium phosphate, pH 6.3, and applied to a pAPTG-Sepharose 4B column (p-aminophenyl- β -D-thiogalactopyranoside) equilibrated with the same buffer (Sigma Chemical Co.). The charged column was washed with 5-10 column volumes of the loading buffer and the toxin eluted by changing the buffer system to 100 mM sodium phosphate, 1.0 M sodium chloride, pH 7.9. Fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by addition of 39 g ammonium sulfate/100 ml.

Electrophoresis.

Protein samples were examined electrophoretically using the Pharmacia Phastsystem (Pharmacia LKB Inc., Piscataway, NJ) according to the manufacturers instructions. Precast 12.5% acrylamide gels (Pharmacia) were stained with 0.1% coomassie brilliant blue R250 in 16.7% acetic acid, 41.7% methanol. Gels were destained in 7.5% acetic acid, 25% methanol. Samples for electrophoresis were solubilized in 50 mM Tris-HCl, 5 M urea, 5% SDS, 20% glycerol, pH 6.8. Some samples were reduced by addition of dithiothreitol to a final concentration of 0.5%. All samples for SDS-PAGE were boiled for ≥ 5 min prior to electrophoresis.

Toxin assays.

Toxin titers were estimated in mice using the intravenous method of Boroff and Fleck (1966) and the intraperitoneal method of Schantz and Kautter (1978) in 18-22 g, female, ICR strain mice. Time-to-death values obtained from intravenous titration of type A and B toxin samples were converted to intraperitoneal LD₅₀/ml using a standard curve generated in our laboratory with type A complex. Botulinum toxin for titration was dissolved in 50 mM sodium phosphate, pH 6.8, and then further diluted as required in 30 mM sodium phosphate, 0.2 % gelatin, pH 6.4.

Lyophilization and excipients.

For lyophilization, toxin samples were diluted in the excipients to be tested (all excipients were from Sigma Chemical Co.), 0.1 ml or 0.5 ml aliquoted into 2 ml glass vials (Fisher Scientific Co., Pittsburgh, PA), the teflon lined screw cap closures fastened loosely, and the samples quickly frozen in liquid nitrogen. The frozen samples were placed into a lyophilization flask which was then immersed in liquid nitrogen. The flask was then connected to a laboratory freeze-drier (Virtis Freezmobile12, Virtis Co., Inc., Gardiner, NY). When the pressure dropped to ca. 30 mTorr, the liquid nitrogen jacket was removed. Pressure was maintained at or below 30 mTorr and condenser temperature was constant at -60°C. Samples were allowed to come to room temperature and drying continued at ambient temperature over the next 18-24 h. At that time the flask was removed and the vials tightly capped. Vials were assayed for toxicity within 1-3 days (adapted from Goodnough and Johnson, 1992).

Some vials of lyophilized type A neurotoxin and type A toxin complex were stored at various temperatures to investigate the effect of added excipients on the shelf-stability of the dried material. In these cases, the tightly capped vials were placed into plastic bags,

sealed and stored at various temperatures (-20, 4, or 37°C) and the contents assayed for toxicity at various time points.

Results

Toxin purification.

Samples of crystalline type A toxin complex electrophoresed on sodium dodecylsulfate-polyacrylamide gels (SDS-PAGE) showed banding patterns typical for type A toxin complex (Figure 1, lanes 1, 2). Chromatographically purified type A toxin complex was electrophoretically equivalent to the crystalline material on SDS-PAGE (Figure 1, lanes 3, 4) and showed a higher specific toxicity than the crystalline toxin (3.2×10^7 LD₅₀/mg for the chromatographed material vs. 2.0×10^7 LD₅₀/mg for the crystalline toxin).

Type A neurotoxin purified from the non-toxic components of the complex showed a single band on unreduced SDS-PAGE gels of ca. 145 kDa indicating that it was free of contaminating non-toxic complex proteins (Figure 2, lane 1). Upon reduction of the disulfide bond with dithiothreitol, the two chains of the toxin migrated separately as the 93 kDa heavy chain and the 52 kDa light chain (Figure 2, lane 2). The purified neurotoxin had a specific toxicity of 9.0×10^7 LD₅₀/mg.

Unreduced SDS-PAGE samples of purified type B neurotoxin showed a single band at ca. 152 kDa indicative of type B neurotoxin (Figure 2, lane 3) (DasGupta and Sugiyama, 1976). Reduced samples showed three bands of ca. 152, 102, and 50 kDa (Figure 2, lane 4). The specific activity of the purified type B neurotoxin was 1.05×10^8 LD₅₀/mg.



Figure 1. SDS-PAGE of *Clostridium botulinum* type A toxin complex purified by the modified method of Duff et al. (1957) (lanes 1 and 2) and by the method of Tse et al. (1982) (lanes 3 and 4). Lane 1- type A toxin complex (crystalline) (unreduced); lane 2- type A toxin complex (crystalline) (reduced with 0.5% w/v dithiothreitol; lane 3- type A toxin complex (chromatographically purified) (unreduced); lane 4- type A toxin complex (chromatographically purified) (reduced), 4-6 μ g protein each lane.



Figure 2. SDS-PAGE of purified type A and B *Clostridium botulinum* neurotoxins. Lane 1- purified type A neurotoxin (unreduced); lane 2- purified type A neurotoxin (reduced with 0.5% w/v dithiothreitol); lane 3- purified type B neurotoxin (unreduced); lane 4- purified type B neurotoxin (reduced), ca. 4 μ g each lane.

Stabilization of botulinum toxin during lyophilization.

Lyophilized preparations were usually reconstituted in 1.0 ml of distilled water. The use of 0.85% saline as a diluent gave equivalent results. The white cake dissolved immediately and was mixed by gentle inversion of the vials. The resulting solution was transparent. This solution was titrated by the same method used for the pre-lyophilization solution. Percent recovery values (calculated as number of mouse IP lethal doses/vial after lyophilization divided by number of mouse IP lethal doses/vial before lyophilization \times 100) represent averages of trials done in at least duplicate. The variation in independent assays was ca. \pm 20% as reported earlier by Schantz and Kautter (1978).

We initially determined whether recovery of active toxin following lyophilization was dependent on the rate of freezing and hence on ice crystal size. Freezing at -20°C or -70°C for 24 h in 50 mM sodium phosphate buffer, pH 6.8, resulted in slight loss of activity (75-90% recovery) compared to $>90\%$ recovery on flash freezing at -200°C in liquid nitrogen followed by 24 h storage at -70°C (data not shown). This could indicate that the larger ice crystals formed in the slower freezing cycle had a slightly negative impact on toxin stability by forming a larger more open structure when these crystals were sublimed during lyophilization. Previous studies in our laboratory also showed that no detectable inactivation ($\leq 20\%$) of type A crystalline toxin (10^4 LD₅₀/ml) occurred during repeated freezing and thawing at -20°C in several buffers. These included 50 mM sodium phosphate (pH 6.2-6.8), 50 mM sodium succinate (pH 6.0), or 50 mM sodium citrate (pH 5.5) (Whitmer, Johnson and Schantz, unpublished). Freezing in 30 mM acetate buffer, pH 4.2, resulted in irreversible loss of toxicity (Schantz and Scott, 1981; Whitmer et al., 1987; Goodnough and Johnson, 1992).

The effect of salt concentration during freezing was examined for its effect on toxicity. Botulinum toxin is precipitated during various purification steps with ammonium sulfate at concentrations in excess of 60% saturation at room temperature with no loss of

activity. This is ca. equivalent to a 33% (w/v) solution of the salt. Sodium chloride at 0.9% in the commercial formulation may reach concentrations in excess of 6 M during lyophilization (Franks, 1990a) or ca. 35% (w/v) prior to crystallization. Freezing samples of type A toxin complex at -20°C and -70°C in 5.0 M solutions of sodium chloride, pH 6.2, did not affect toxin activity and full recovery was obtained after 24 h at both freezing temperatures and rates tested.

• Lyophilization of type A toxin complex at 100-1,000 LD₅₀/vial (i.e. 3.3-33 ng/vial) in the absence of protein drying excipients gave almost complete loss of activity (Table 1, adapted from Goodnough and Johnson, 1992). It was not possible with such small quantities of toxin to determine if there were any losses due to aggregation since no visible precipitate formed when the dried material was reconstituted. The recovery of botulinum toxin activity following lyophilization was dependent on three factors. The formulation had to have a protein stabilizing agent present. In our case, serum albumins were most commonly used although other globular proteins such as gelatin, alpha-lactalbumin, and lysozyme worked in place of the albumins. The preparation for drying had to be free of sodium chloride and the pH of the preparation had to be maintained below 7.0 (Table 1). The results which stemmed from studies using crystalline type A toxin complex were then applied to other forms of botulinum toxin.

Type A toxin complex which was chromatographically purified gave identical recovery as the crystallized type A toxin complex following lyophilization. Chromatographically purified type B toxin complex (a mixture of the 300 kDa M and 500 kDa L complexes) was lyophilized under conditions which were favorable for recovery of type A toxin activity. Recovery of the chromatographically purified type B complex was the same as that attained with type A toxin complex (Table 2).

Table 1. Effect of excipients on recovery of toxicity of *Clostridium botulinum* type A toxin complex after lyophilization.

| Excipients | Starting Toxin concentration ^a | pH | %recovery ^b |
|---|---|---------------|------------------------|
| sodium phosphate ^c | 50, 100, 1,000 | 5.0, 6.0, 6.8 | <10 |
| bovine serum albumin/ sodium chloride ^d | 100 | 6.4 | 10 |
| bovine serum albumin ^e | 100, 1,000 | 6.4 | 88, 75 |
| bovine serum albumin/citrate ^f | 100, 1,000 | 5.0 | >90, >90 |
| bovine serum albumin/phosphate ^g | 100, 1,000 | 5.5 | >90, >90 |
| bovine serum albumin/phosphate ^g | 1,000 | 7.3 | 60 |
| bovine serum albumin/phosphate ^h | 1,000 | 6.0 | >90 |
| human serum albumin ⁱ | 100, 1,000 | 6.4-6.8 | >90, >90 |
| alpha-lactalbumin ^j | 1,800 | 6.1 | >78 |
| lysozyme ^j | 1,800 | 5.3 | >78 |
| gelatin ^j | 1,800 | 6.3 | >78 |
| bovine serum albumin/ trehalose ^k | 500 | 5.7 | >90 |
| bovine serum albumin/ sucrose ^l | 325 | 6.6 | 65 |
| bovine serum albumin/ maltotriose ^m | 250 | 7.0 | >80 |

^a Type A mouse IP lethal doses/vial before lyophilization; ^b %recovery = (number mouse lethal doses after lyophilization/number mouse lethal doses prior to lyophilization) x 100;

^c 50mM sodium phosphate; ^d bovine serum albumin (5.0mg/ml), sodium chloride (9.0mg/ml); ^e bovine serum albumin (9.0mg/ml); ^f bovine serum albumin (9.0mg/ml), 50mM sodium citrate; ^g bovine serum albumin (9.0mg/ml), 50mM sodium phosphate; ^h bovine serum albumin (9.0mg/ml), 50mM potassium phosphate; ⁱ human serum albumin (9.0mg/ml); ^j concentration = 9.0mg/ml; ^k 9.0mg/ml bovine serum albumin, 100mg/ml trehalose; ^l 9.0mg/ml bovine serum albumin, 250mg/ml sucrose; ^m 9.0mg/ml bovine serum albumin, 100mg/ml maltotriose. (adapted from Goodnough and Johnson, 1992).

Table 2. Recovery of activity following lyophilization of *Clostridium botulinum* type B toxin complex^a.

| Excipient combination | Starting toxin concentration ^b | pH | %recovery ^c |
|---|---|----------|------------------------|
| bovine serum albumin/phosphate ^d | 1,000; 100 | 6.0 | >90 |
| bovine serum albumin/phosphate ^e | 1,000 | 6.0 | >90 |
| bovine serum albumin ^f | 1,000 | 6.4, 6.8 | >90 |
| human serum albumin ^g | 1,000 | 6.4 | >90 |
| gelatin ^h | 1,000 | 6.2 | >90 |

^a type B complex was a mixture of 300 kDa and 500 kDa complexes (Sakaguchi, 1983);

^b mouse intraperitoneal lethal doses/vial; ^c (number of mouse lethal doses/vial after lyophilization + number of mouse lethal doses before lyophilization) x 100; ^d 9.0mg/ml bovine serum albumin, 50mM sodium phosphate; ^e 9.0mg/ml bovine serum albumin, 50mM potassium phosphate;

^f 9.0mg/ml bovine serum albumin; ^g 9.0mg/ml human serum albumin; ^h concentration = 9.0mg/mL.

In further efforts to reduce the amount of neurotoxin needed to yield 100 LD₅₀/vial following lyophilization, purified type A and B neurotoxins were dried in the presence of serum albumin. Although the neurotoxins are more labile than the complexes, recoveries on drying were similar to those obtained with the complexes (Table 3). The quantities of neurotoxin required to attain 100 LD₅₀ of active toxin following lyophilization were ca. 1 ng per vial as compared to 3-8 ng per vial of the type A and B complexes. Recoveries of type A and B neurotoxin following lyophilization were high (≥80% of pre-lyophilized starting values) except in the samples which sucrose was added at a concentration of 250 mg/ml. The lower recovery rate (50%) with sucrose may have been related to a higher level of residual moisture as solutions containing high concentrations of sucrose are difficult to dry completely (Franks, 1990a). This residual moisture also had a deleterious effect on activity of selected type A botulinum toxin preparations when the different toxin preparations were stored at elevated temperatures (above -20°C) (Figures 3 and 4).

Table 3. Recovery of activity following lyophilization of purified *Clostridium botulinum* type A and B neurotoxins.

| Excipient combination/ neurotoxin type ^a | Starting toxin concentration ^b | pH | %recovery ^c |
|--|--|-----|------------------------|
| bovine serum albumin/ type A | 200 | 6.4 | 90 |
| bovine serum albumin/ type B | 100 | 6.4 | 80 |
| human serum albumin/ type A | 1,000 | 6.4 | 90 |
| human serum albumin/ type B | 100 | 6.4 | 90 |
| bovine serum albumin, trehalose/ type A | 500 | 5.7 | >90 |
| bovine serum albumin, sucrose/ type A | 325 | 6.6 | 50 |
| bovine serum albumin, maltotriose type A | 250 | 7.0 | 90 |

^a bovine and human serum albumin concentration was 9.0 mg/ml, carbohydrate concentration was 100 mg/ml in all cases except sucrose which was 250 mg/ml; ^b mouse intraperitoneal lethal doses/vial; ^c (number of mouse lethal doses/vial after lyophilization ÷ number of mouse lethal doses before lyophilization) x 100.

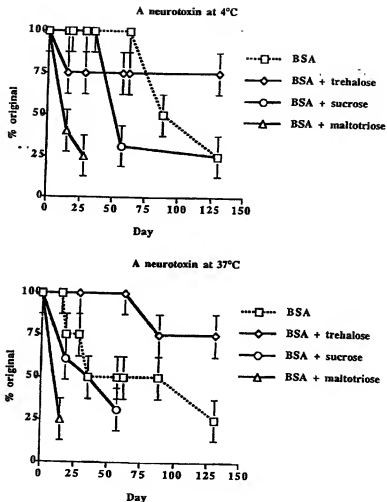


Figure 3. Stability of lyophilized purified type A neurotoxin at 4 and 37°C in the presence of bovine serum albumin and carbohydrate excipients.

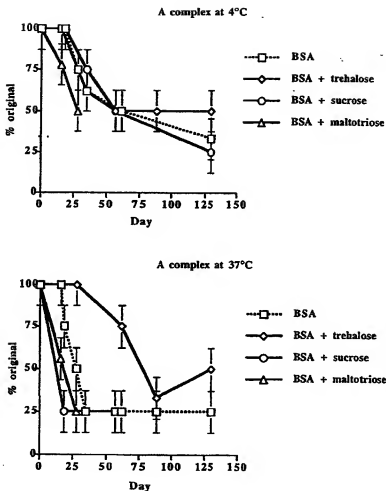


Figure 4. Stability of tyophilized type A toxin complex at 4 and 37°C in the presence of bovine serum albumin plus various carbohydrate excipients.

Stability of lyophilized botulinum toxin during extended storage.

Three different carbohydrate excipients were added to the bovine serum albumin system in an effort to raise the shelf-storage temperature by elevating the glass transition temperature of the dried material. Trehalose and maltotriose were added to the drying formulation at 100 mg/ml while sucrose was added at 250 mg/ml. Purified type A neurotoxin and crystalline type A toxin complex were used in separate experiments. The results with purified neurotoxin indicated that trehalose had a stabilizing effect at 4 and 37°C (75% activity retained after 130 days storage) which was much greater than the other two carbohydrates tested (<25% activity retained for each) (Figure 3). Type A crystalline toxin complex was also stabilized by trehalose at the elevated shelf temperatures tested (50% retained activity after 130 days), but not to the same degree as that obtained with purified type A neurotoxin. Control vials for each combination of excipients were stored at -20°C and did not show any reduction in potency over the 130 days of incubation.

Discussion

The conditions used for lyophilization in this study had a considerable effect on recovery of active botulinum toxin. One of the most critical factors that contributed to recovery of active toxin was the omission of sodium chloride from the solution to be lyophilized. This omission combined with the presence of a protein excipient in large excess (1,000-10,000 fold excess) of the botulinum toxin concentration yielded >90 % of the starting toxicity when the pH was maintained below 7.0. Recovery of toxin activity following lyophilization was dependent to a more limited extent on the pH of the solution prior to lyophilization (Table 1). pH values of 7.0 and 7.3 were tested which did not give recovery rates as high as those obtained at pH values below neutral (Table 1). While freeze concentration and subsequent differential crystallization rates of the buffer components and salts present in solution, especially for sodium phosphate buffered systems, has been shown to alter the pH during lyophilization (Van den Berg, 1966; Pikal, 1990), the use of sodium phosphate or potassium phosphate in our experiments did not effect recovery of active type A toxin complex (Table 1). Solutions of BSA or HSA at 9.0 mg/ml had pH values of 6.4-6.8 at which full recovery was obtained. Full recovery of toxin activity was also obtained when the pH was adjusted to 5.0 or 5.5 by the use of sodium phosphate or sodium citrate buffered systems (Table 1). Recovery of activity following lyophilization of purified type A and B neurotoxin does not seem to be dependent on the presence of the non-toxic binding proteins of the complex as a high percentage of toxin activity was recovered using the same formulation as that used for the type A and B toxin complexes.

The differences in recovery rates dependent on pH could be due to the tendency for increased deamidation at higher pH levels. Deamidation with loss of activity has been demonstrated with other proteins such as lysozyme (Ahern and Klivanov, 1985), triose-phosphate isomerase (Ahern et al., 1987), calmodulin (Johnson et al., 1989a) and others

(Johnson et al., 1989b). Asparagine deamidates more readily than glutamine in model peptides with contributing factors being the primary amino acid sequence and the tertiary structure of the protein (Wright, 1991; Liu, 1992). Also, deamidation is more prevalent at asparagine-glycine and asparagine-serine sequences than at other asparagine sequences (Liu, 1992; Wright, 1991). There are at least six asparagine-glycine sequences in type A neurotoxin with three being in the light chain and three in the heavy chain. There are eight asparagine-glycine sequences present in the heavy chain region of the toxin (Binz et al., 1990). In other protein systems, these sequences were deamidated under certain drying and storage conditions (Wright, 1991). Further work will be done to determine whether deamidation is occurring in botulinum toxin during lyophilization and storage.

In most experiments in this study, 0.1 ml of botulinum toxin was lyophilized. This is equal to or less than the amount needed for most automated filling equipment in commercial lyophilization laboratories. When the fill volume was increased to 0.5 ml (with a subsequent reduction in serum albumin concentration to 1.8 mg/ml to maintain a post-lyophilization concentration of 0.9 mg/ml following reconstitution) slightly lower recoveries (60-80% of initial toxicity) were obtained on lyophilization (Goodnough and Johnson, 1992). This could be due to the fact that the same size 2 ml vials and lyophilization cycle were used causing the frozen cake to be thicker. This increased resistance to water vapor escape could have left more residual moisture in the freeze dried cake since the same lyophilization cycle was employed.

Shelf stability is an important property of protein pharmaceuticals. The present formulation of botulinum toxin must be stored below -10°C and ideally below -20°C following drying to retain potency of the dried toxin. One possible reason for the instability observed at ambient temperatures is that the glass transition temperature of human serum albumin and sodium chloride is around -10°C. Storage above this transition temperature would allow the residual moisture in the amorphous phase to interact with the

toxin molecule promoting degradative chemical reactions (Franks, 1990b). The glass transition temperatures of commercially dried pharmaceuticals containing protein can be elevated by the addition of certain carbohydrates. For instance, Franks (1990b) lists the glass transition temperatures of trehalose, sucrose, and maltotriose as 77°C, 57°C, and 76°C respectively. From these data one could assume that the choice of carbohydrate excipient would not be critical. However, the water contents on freeze-drying these carbohydrates are significantly different. Trehalose retains the lowest with 16.7% water followed by maltotriose (31.0%) and sucrose (35.9%) (Franks, 1990b). The higher percentage of water associated with the freeze-concentrated material would require a longer drying cycle to remove this water. The majority of the moisture in the initial formulation is removed relatively quickly during the primary drying portion of the lyophilization cycle. It is during this part of the cycle that the ice crystals formed during freezing are sublimed. Remaining moisture such as that associated with water of hydration and bound in crystalline structures must be removed in the much longer secondary drying portion of the lyophilization cycle.

One aspect of the shelf life of botulinum toxin complex which has not previously been investigated is whether the presence of the non-toxic binding proteins add stability to the toxin molecule in the lyophilized state. These non-toxic binding proteins add stability to the toxin molecule in solution and in the gastric tract. Buffered solutions of purified neurotoxin show breakdown fragments and a reduction in specific activity when incubated in solution at ambient temperature in only a few days. Solutions of toxin complex do not show these breakdown fragments and maintain specific activity for weeks (Goodnough and Johnson, unpublished data).

Our results indicate that purified type A neurotoxin is more stable at elevated temperatures than type A toxin complex when lyophilized in the three excipient systems tested. One possible explanation for this is that when toxin complex is lyophilized there is

more residual water present in close proximity to the toxin molecule itself due to the water of hydration from the non-toxic binding proteins of the complex. This water which is already in close proximity to the toxin molecule could be available for degradative reactions when the dried material is raised above its glass transition temperature. Another possible explanation is that the type A toxin complex preparations had a small amount of a protease(s) which copurified with the toxin complex while these contaminants were removed during purification of the neurotoxin. These degradative enzymes could inactivate the toxin when there was sufficient free water present. The pure form of the toxin was much more extensively chromatographed and could have had these low level contaminants removed. As a third possibility, one of the non-toxic binding proteins themselves could have some kind of enzymatic properties which may have contributed to the inactivation of the lyophilized complex.

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CHAPTER VI

Mechanisms of inactivation of Clostridium botulinum neurotoxin
during lyophilization

Abstract

Treatment of hyperactive muscle disorders with the neurotoxin of Clostridium botulinum involves direct injection of afflicted muscle groups with the reconstituted toxin. Prior to distribution to clinicians, the toxin must be carefully quantitated and dispensed then freeze-dried or lyophilized to allow shipment and handling of this delicate protein. The various drying processes and formulations cause varying degrees of inactivation of the toxin and formation of toxoid. This toxoid adds to the antigenic burden of the material and further increases the chances of patients developing neutralizing antibodies. The biochemical processes which result in the formation of this toxoid can involve aggregation, deamidation, peptide bond hydrolysis, and oxidative degradation. In this study, we demonstrate in a model system using purified type A and B Clostridium botulinum neurotoxins that aggregation, deamidation, and peptide bond hydrolysis occur during lyophilization procedure causing decreases in the specific toxicity.

Introduction

Clostridium botulinum is a heterogeneous group of organisms that produce a very potent proteinaceous neurotoxin which binds to motor-neurons at presynaptic junctions and inhibits the release of acetylcholine causing a flaccid paralysis (Schantz and Johnson, 1992). The organisms comprising the group are characterized by differing degrees of proteolysis and by the serotype of neurotoxin produced. There are seven known serotypes A, B, C1, D, E, F, and G. The neurotoxin is post-translationally processed to form the active dichain molecule. Post-translational "nicking" occurs ca. one-third the length of the protoxin molecule from the N-terminus generating two fragments connected by a disulfide bond. This dichain molecule is comprised of a light chain (ca. 50 kDa) which is the neurotoxic portion and a heavy chain (ca. 100 kDa) which is responsible for binding to the receptor on the motor-neuron and internalization of the light chain (Niemann, 1991). The exploitation of the flaccid paralysis caused by type A toxin has been used in the treatment of spastic muscle disorders in humans since about 1981 (Schantz and Johnson, 1992). The U.S. Food and Drug Administration licensed type A botulinum toxin for treatment of some of these disorders as an orphan drug in 1989.

Freeze-drying or lyophilization has become a standard method of stabilizing proteins which are susceptible to inactivation or possible microbial contamination when stored in dilute solutions. Freeze-drying subjects proteins to changes in temperature, increases in salt concentration, alterations in pH, and exposure to degradative reactions (Pikal, 1990; Franks, 1990; Liu, 1992). It has been shown that exposure of C. botulinum toxin to high salt concentrations or to low pH values during purification is not detrimental to toxin activity (Schantz, 1964; Schantz and Johnson, 1992). However, the neurotoxin is a fragile protein molecule and has been shown to fragment under relatively mild conditions (DasGupta and Tepp, 1991). Exposure of some pharmaceutical peptides to mildly alkaline

pH values and moderate temperatures leads to deamidation of the primary amino acid sequences of some pharmaceutical peptides (Johnson and Aswad, 1990; Johnson et al., 1989a; Johnson et al., 1989b; Manning et al., 1989). Asparagine and glutamine residues can react with succeeding glycine residues at physiologic pH values (pH 6-8) leading to the formation of a cyclic imide structure with the corresponding loss of an amine group from the side chain of the asparagine or glutamine. The cyclic imide can then break open to form a normal L-aspartic or glutamic acid linkage or the D-isomer of the aspartic or glutamic linkage. Isoelectric focusing and cation exchange has been used with model peptides or relatively small proteins to show differences in overall charge due to deamidation (Liu, 1992; Patel, 1993).

Other biochemical processes can lead to denaturation of protein pharmaceuticals during lyophilization. Aggregation of protein can occur during freeze-drying due to exposure of internal hydrophobic amino acid residues to the aqueous solvent and to hydrophobic surfaces such as at solvent-air interfaces (Sluzky et al., 1991). Under such conditions, the formation of intermolecular hydrophobic interactions is favored which causes aggregation of the protein.

Hydrolysis can also cause loss of activity of proteins during lyophilization. The peptide bond between aspartate and proline residues can hydrolyze at low pH values and ambient temperatures (Marcus, 1985). The hydrolysis of certain aspartate-proline sequences in the heavy chain of type A *C. botulinum* neurotoxin occurred at pH values as high as 5.0 (DasGupta and Tepp, 1991).

The presence of free water is necessary in all of these denaturation reactions. In lyophilized protein preparations, free water is generally present only at temperatures above that of the glass transition temperature of the material (Franks, 1990). The glass transition is the temperature at which the material changes from an elastic solid or glass, in which the water is held in place (i.e. only very slowly diffusible), to that of a more pliable or

deformable rubber material in which water is free to diffuse and participate in chemical reactions. The rate of these reactions is dependent on the final water content of the dried material as well as the glass transition temperature of the components in the formulation (Franks, 1990).

In this chapter, I have examined processes which cause inactivation of botulin toxin during lyophilization. This work suggests that the drying formulation and process may be optimized to reduce inactivation and formation of toxoid.

Bacterial culture and toxin purification.

Clostridium botulinum Hall A strain was used for the production of type A toxin complex. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 h). *C. botulinum* Okra B strain from the Food Research Institute culture collection was used to produce type B toxin complex. Stock cultures of each organism were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3 % dextrose (CMM, (Difco Laboratories, Detroit, MI)) under an anaerobic atmosphere (80% N₂, 10%CO₂, 10%H₂) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain gave toxin titers in excess of 10⁶ intraperitoneal 50% lethal doses (LD₅₀/ml) in 18-22g white mice within 48-72 h while Okra B cultures gave toxin titers of 5-9 x 10⁵ LD₅₀/ml of crude culture.

For toxin production, cultures of Hall A and Okra B were grown statically in 12-15 liter volumes of toxin production medium (TPM) consisting of 2.0% casein hydrolysate (Sheffield Laboratories, Norwich, NY), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37°C. Cultures showed heavy growth in this medium during the first 24-48 h followed by autolysis of the cultures which was evident as a clearing and settling over the next 48-120 h.

Toxin purification.

Crystalline type A toxin complex was purified by a modification of the method of Duff et al. (1957) as described elsewhere (Schantz, 1964; Goodnough and Johnson, 1992; Schantz and Johnson, 1992). This method was identical to the method used to produce type A toxin complex for medical use in the United States. Purified type A neurotoxin was

purified by the method of Tse et al. (1982) with the addition of a final chromatographic step on SP-Sephadex C50 (Sigma Chemical Co., St. Louis, MO) at pH 7.0 according to the method of DasGupta and Sathyaamoorthy (1984).

Type B neurotoxin was purified from crude culture by a method involving the chromatographic procedures of Tse et al. (1982) and Moberg and Sugiyama (1978). Briefly, cultures of *C. botulinum* Okra B were acid precipitated to collect the toxin from the whole culture. Type B toxin complex was separated from the remaining proteins in the extract of the acid precipitate on a 1 liter DEAE-Sephadex A50 (Sigma Chemical Co., St. Louis, MO) (5 cm x 65 cm) at a pH of 5.5 in 50 mM sodium citrate buffer. The neurotoxin was partially separated from the associated non-toxic proteins of the complex on a DEAE-Sephadex A50 column at pH 7.9 according to the method of Tse et al. (1982). The partially purified neurotoxin was purified to homogeneity by binding the non-toxic proteins of the complex to a pAPTG-Sepharose 4B column (p-aminophenyl- β -D-thiogalactopyranoside) (Sigma) and eluting the purified toxin as a single peak according to the method of Moberg and Sugiyama (1978).

Electrophoresis.

Protein samples were examined by SDS-PAGE using the Pharmacia Phastsystem and precast, linear 12.5% polyacrylamide gels (Pharmacia LKB Inc., Piscataway, NJ) according to the manufacturers instructions. SDS-PAGE was also done with the BioRad Protean II system (BioRad Laboratories, Richmond, CA) using linear polyacrylamide gels according to the method of Laemmli (1970) as modified by Hames (1990). Gels were stained with 0.1% coomassie brilliant blue R250 in 16.7% acetic acid, 41.7% methanol and destained in 7.5% acetic acid, 25% methanol. Some gels were further silver stained according to the method Hames (1990). Protein samples for SDS-PAGE were solubilized in 50 mM Tris-HCl, 5 M urea, 5% SDS, and 20% glycerol, pH 6.8. Some samples were

reduced by addition of dithiothreitol to a final concentration of 0.5% (w/v). Samples for SDS-PAGE were boiled for 5-10 min prior to electrophoresis.

Proteolytic digestion and peptide mapping was done according to the method of Cleveland et al. (1977). For this analysis, protein bands were excised from the first SDS-gel after staining with Coomassie blue. The bands were cut out of the first gel using a razor blade and loaded directly onto the second gel.

Samples for native gel analysis were solubilized in 50 mM Tris-HCl, 20% glycerol, pH 6.8, and were run on pre-cast, 4-15% gradient gels according to manufacturers instructions (Pharmacia). Urea was added to some samples for native gel electrophoresis to a final concentration of 8 M. Samples for isoelectric focusing were dissolved and diluted in 25 mM sodium phosphate, pH 7.3, and run using pre-cast isoelectric focusing gels (Pharmacia) according to the manufacturers instructions.

Electrotransfer of SDS-PAGE gels for amino acid sequencing.

Proteins separated by SDS-PAGE using the BioRad Protean II system were transferred to Immobilon brand PVDF (polyvinylidene difluoride) membrane (Millipore Corp., Bedford, MA) according to the method of Matsudaira (1987). Transfers were made onto PVDF membranes in 10 mM CAPS (3-cyclohexylaminopropanesulfonic acid), 5% methanol, pH 10, at 30 mA constant current overnight (ca. 16 h) using the BioRad Transblot electrotransfer cell. The PVDF membranes were first briefly wetted with 100% methanol prior to assembly of the electrotransfer cassette. Following transfer, membranes were briefly (2-3 min) stained with 0.1% Coomassie brilliant blue R250 in 40% methanol and destained with 40% methanol to visualize transferred bands. Bands for amino acid sequencing were excised with a clean razor blade and sequenced at the University of Wisconsin Biotechnology Center, Madison, WI.

Toxin assays.

Toxin titers were estimated in mice using the intravenous method of Boroff and Fleck (1966) and the intraperitoneal method of Schantz and Kauter (1978) in 18-22 g, female, ICR strain mice. Time-to-death values obtained from intravenous titration of type A and B toxin samples in 3-5 mice were averaged and converted to intraperitoneal LD₅₀/ml using a standard curve generated in our laboratory for the type A complex. Botulinum toxin for titration was dissolved in 50 mM sodium phosphate, pH 6.8, and then further diluted as required in 30 mM sodium phosphate, 0.2 % gelatin, pH 6.4.

Deamidation assays.

Estimation of the isoaspartyl content of unlyophilized or lyophilized, and reconstituted purified A and B neurotoxins was done using a methanol diffusion assay of Macfarlane (1984) as modified by McFadden and Clarke (1986). Isoaspartyl-delta sleep inducing peptide (isoAsp-DSIP) (BACHEM Bioscience, Inc., Philadelphia, PA) was used as a standard. IsoAsp-DSIP has the sequence:



where Asp* is the residue with the isopeptide bond.

Isoaspartyl residues were methylated in a reaction volume of 50 μ l at 30°C for 30 min in the presence of 2 μ M protein methyltransferase (protein L-isoaspartyl methyltransferase type II; PIMT; E.C. 2.1.1.77 kindly provided by Dr. D. Aswad, University of California, Irvine) and 50 μ M S-adenosyl-L-[methyl-³H] methionine (500-600 dpm/pmol). The specific activity of the PIMT was 15-20 nmol/min/mg at 30°C using gamma-globulin as substrate or 25-30 nmol/min/mg using isoaspartyl-delta sleep inducing peptide isoAsp-DSIP. Methylation reactions were stopped by addition of 50 μ l of 0.4 M

sodium borate, 5% SDS, 2.2% methanol, pH 10.0, and vortexing thoroughly. Twenty-five μ l of individual reactions were spotted onto filter paper in the caps of 20 ml scintillation vials containing 10 ml of Ecolume (ICN, Costa Mesa, CA), the caps tightened, and the vials incubated at 40°C for 1 h. At that time the caps containing filter paper were replaced with new caps and the vials counted in a Beckman LS 5801 liquid scintillation counter.

Protein concentration determination

Toxin concentrations were estimated using the extinction coefficients for type A toxin complex of A278 1.65, purified type A neurotoxin of A278 1.63 (Knox et al., 1970) and for both type B toxin complex and purified type B neurotoxin of A278 1.85 = 1 mg/ml in a 1 cm light path (Beers and Reich, 1969). Protein concentrations were also estimated using the bicinchoninic acid method of Smith et al. (1985) with bovine serum albumin as the standard.

Amino acid sequencing

Amino acid sequencing was done at the University of Wisconsin Biotechnology Center using an automated model 477A Liquid Pulse sequencer with on-line model 120A PTH analyzer and 610A data analysis system (Applied Biosystems, Foster City, CA).

Size exclusion high pressure liquid chromatography

HPLC was performed with a Rainin HPXL system (Rainin Instrument Co., Woburn, MA). The size exclusion columns used were a Rainin SEC column (4.6mm x 250 mm) at a flow rate of 0.25 ml/min or a Dupont Zorbax GF-450 column (9.4 mm x 250 mm) at a flow rate of 1.0 ml/min. The isocratic solvent system in both cases was 100 mM sodium phosphate, pH 7.0, and protein was detected by absorbance at 278 nm. The

molecular weight cutoff for both columns was reported by the manufacturers to be $1-2 \times 10^3$ kDa.

Results

SDS-PAGE of botulinum toxin preparations.

The purity of type A toxin was evaluated by SDS-PAGE (Figure 1). Lane 2 contained 4-6 µg of purified type A neurotoxin with a specific toxicity of 96 LD₅₀/ng. A single major band is seen at 145 kDa. Lane 3 contained 4-6 µg of purified type A neurotoxin that was reduced with 0.5% (w/v) dithiothreitol. Two major bands are seen at 93 kDa (heavy chain) and 52 kDa (light chain). Lane 4 contained 4-6 µg of type A toxin complex and showed the characteristic 7 bands. Lane 5 contained 4-6 µg of type A toxin complex that was reduced with 0.5% (w/v) dithiothreitol.

Purified type B neurotoxin was analyzed by SDS-PAGE (Figure 2). Lanes 1 and 2 contained crystalline type A toxin complex (unreduced and reduced, 4-5 µg each lane) which was used as a molecular weight marker. Lane 3 contained 5-6 µg of purified type B neurotoxin, molecular weight ca. 152 kDa (unreduced). Lane 4 contained 5-6 µg of purified type B neurotoxin that was reduced with dithiothreitol (0.5% w/v) showing heavy (ca. 102 kDa) and light (ca. 50 kDa) chains. In contrast to type A, a substantial portion of type B neurotoxin remained in the unnickd form (152 kDa) after reduction of the disulfide bond connecting the individual chains.

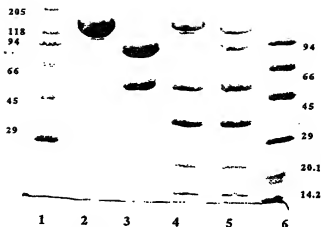


Figure 1. SDS-PAGE analysis of type A toxin preparations. Lane 1, molecular weight markers (in kDa); rabbit myosin, 205, *E. coli* β -galactosidase, 118, *E. coli* phosphorylase, 94, bovine serum albumin, 66, ovalbumin, 45, carbonic anhydrase, 29, (Sigma Chemical Co., St. Louis, MO), 5-6 μ g protein total; lane 2, purified type A neurotoxin (unreduced), 4-5 μ g protein; lane 3, purified type A neurotoxin (reduced with 0.5% (w/v) dithiothreitol), 4-5 μ g protein; lane 4, type A toxin complex (unreduced), 4-5 μ g protein; lane 5, type A toxin complex (reduced), 4-5 μ g protein; lane 6, molecular weight markers (in kDa); *E. coli* phosphorylase, 94, bovine serum albumin, 66, ovalbumin, 45, carbonic anhydrase, 29, soybean trypsin inhibitor, 20.1 (doublet), and alpha lactalbumin- 14.2, (Pharmacia), 4-6 μ g total.

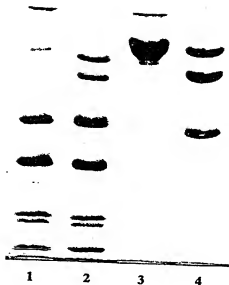


Figure 2. SDS-PAGE of type A toxin complex and purified type B neurotoxin. Lane 1, crystalline type A toxin complex (unreduced), 4-5 μ g protein; lane 2, crystalline type A toxin complex (reduced with 0.5% (w/v) dithiothreitol), 4-5 μ g protein; lane 3, purified type B neurotoxin (unreduced), 4-5 μ g protein; lane 4, purified type B neurotoxin (reduced), 4-5 μ g protein.

Aggregation of type A toxin during lyophilization.

Purified type A neurotoxin aggregated when lyophilized in the absence of protein (Table 1). In trial 1 Table 1, toxin was freeze-dried and stored at room temperature for several weeks before being reconstituted. In trials 2 and 3, aggregation was determined within 1-2 days following lyophilization. All trials represent the average of two separate determinations. The data showed that aggregation occurred as indicated by a decrease in soluble protein.

Native gel electrophoresis (Figure 3) of type A complex and purified type A neurotoxin supported the idea that aggregation occurred during lyophilization. Type A toxin complex (lane 1) migrated as a single polypeptide on 4-15% polyacrylamide native gels. When 8M urea was added to the toxin complex, some dissociation occurred (approximately 30%) (lane 2). Purified type A neurotoxin that had been lyophilized and treated with 8 M urea migrated as a single polypeptide. Purified type A neurotoxin that had been lyophilized but not treated with urea contained aggregates that did not enter the gel (lane 4).

Size exclusion chromatography of type A toxin complex by HPLC in 100 mM sodium phosphate, pH 7, at room temperature showed that unlyophilized or lyophilized toxin complex eluted with the same retention time. Following lyophilization, peaks indicative of breakdown products were not detected and peak broadening indicative of aggregation were not observed. However, since the toxin complex (900 kDa) was very nearly at the size exclusion limit of both commercially available columns (approximately 1×10^3 kDa in each case) it may not have been possible to detect large molecular weight aggregates using this system.

Table 1. Losses of purified type A neurotoxin on lyophilization.*

| Sample | Trial | Specific activity ^a | Concentration ($\mu\text{g/ml}$) as determined by: | |
|-----------------------------|-------|--------------------------------|---|------------------|
| | | | A ₂₇₈ ^b | BCA ^c |
| pre- lyoph | 1 | 88 | 100 | 131 |
| | 2 | 96 | 300 | 354 |
| | 3 | 94 | 200 | 220 |
| post- lyoph ^d | 1 | 60 (68%) | 65 (65%) | 80 (61%) |
| | 2 | 80 (83%) | 220 (73%) | 237 (67%) |
| | 3 | 75 (80%) | 173 (87%) | 175 (80%) |

*In 50 mM sodium phosphate, pH 6.8, 0.10 ml fill volume. ^aLD₅₀/ng determined by i.v. method of Boroff and Fleck (1966); ^babsorbance at 278 nm using an extinction coefficient for type A toxin of E_{1%}=16.3 in 1 cm light path; ^cBCA assay (bicinchoninic acid assay with an average of two determinations) using bovine serum albumin as standard (Smith et al., 1985) (Pierce Biochemicals, Rockford, IL, U.S.A.); ^dassayed after being dissolved in 1.0 ml dH₂O and centrifuged to remove aggregated protein. Numbers in brackets represent recovery percentages compared to pre-lyophilized values.



Figure 3. Native gel electrophoresis of unlyophilized type A complex and lyophilized, purified type A neurotoxin on 4-15% polyacrylamide gradient gel. Lane 1, unlyophilized type A toxin complex, 4-5 μ g; lane 2, unlyophilized type A toxin complex plus 8M urea added to the sample buffer prior to electrophoresis, 4-5 μ g; lane 3, lyophilized, purified type A toxin treated with 8 M urea prior to electrophoresis, 2-3 μ g; lane 4, lyophilized, purified type A neurotoxin which was not exposed to urea, 4-5 μ g; lane 5, markers consisting of thyroglobulin, ferritin, and catalase.

Deamidation of type A and B neurotoxins.

Deamidation was investigated as a source of denaturation using the vapor diffusion method of McFadden and Clarke (1986). Assays for deamidation showed that purified type B neurotoxin which had been lyophilized was a better substrate for the PIMT enzyme than its unlyophilized control (Table 2). Purified type A neurotoxin did not show this pattern and there was incorporation of radiolabel in both the unlyophilized and the lyophilized type A neurotoxin samples at approximately the same rate.

Isoelectric focusing was used in an attempt to determine differences in the charge of lyophilized toxin possibly due to deamidation during lyophilization. Purified type A toxin (Figure 4, lane 3) had a specific toxicity of ca. 90 LD₅₀/ng and a pI of 6.1. Upon lyophilization, there was no detectable shift in the isoelectric point of the toxin but the specific toxicity dropped to 65 LD₅₀/ng (Figure 4, lane 4).

Table 2. Incorporation of tritiated methyl groups by protein isoaspartyl methyltransferase into lyophilized and unlyophilized purified type A (Ant) and B (Bnt) neurotoxin.

| Assay # | $\mu\text{M}(\text{toxin})$ | $\text{mol } ^3\text{CH}_3/\text{mol toxin}$ | |
|---------|-----------------------------|--|----------------------------|
| | | Ant ^a | Ant (lyoph'd) ^b |
| 1 | 17.9 | 0.715 | |
| | 20.3 | | 0.613 |
| 2 | 13.6 | 2.160 | |
| | 13.0 | | 2.345 |
| 3 | 13.3 | 2.088 | |
| 4 | 33.9 | Bnt ^c | Bnt (lyoph'd) ^d |
| | 40.5 | 0.110 | 0.290 |
| 5 | 14.5 | 1.230 | |
| | 8.5 | | 3.689 |

^apurified type A neurotoxin (unlyophilized), ^bpurified type A neurotoxin (lyophilized, reconstituted with dH₂O, specific activity = 65 LD₅₀/ng), ^cpurified type B neurotoxin (unlyophilized), ^dpurified type B neurotoxin (lyophilized, reconstituted with dH₂O, specific activity = 53 LD₅₀/ng).

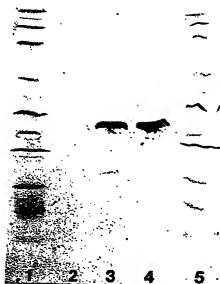


Figure 4. Isoelectric focusing gel (pH 3-9) of lyophilized and unlyophilized purified type A neurotoxin. Lanes 1, 5; broad pI markers (from the top of the gel down) consisting of trypsinogen, 9.30, lentil lectin-basic band, 8.65, lentil lectin-middle band, 8.45, lentil lectin-acidic band, 8.15, horse myoglobin-basic band, 7.35, horse myoglobin-acidic band, 6.85, human carbonic anhydrase B, 6.55, bovine carbonic anhydrase B, 5.85, β -lactoglobulin A, 5.20, soybean trypsin inhibitor, 4.55, and amyloglucosidase, 3.50 (Pharmacia), 8-10 μ g protein; lane 2, sample buffer (25 mM potassium phosphate, pH 6.9); lane 3, purified type A neurotoxin, 4-5 μ g protein; lane 4, lyophilized, purified type A neurotoxin, 4-5 μ g protein.

Peptide bond hydrolysis.

Examination of type A toxin complex by SDS-PAGE before and after lyophilization indicated that no peptide bonds were broken (Figure 5). Lanes 1 (unreduced) and 2 (reduced with 0.5% w/v dithiothreitol) show the banding pattern of unlyophilized toxin complex. The pattern is typical of type A toxin complex showing toxin (lane 1, unreduced, 145 Kda, lane 2, reduced, 93 and 52 kDa) and the associated non-toxic binding proteins (118, 50, 35, 21.8, 20.8, and 17.5 kDa). Lanes 4 (unreduced) and 5 (reduced) show the pattern of polypeptides in the complex following lyophilization. The formulation used for drying contained 50 mM sodium phosphate, pH 7.3, 9.0 mg/ml sodium chloride, and 250 µg/ml of toxin complex with a specific activity of 28 LD₅₀/ng; 0.1 ml was lyophilized in 2 ml glass vials (Goodnough and Johnson, 1992). The lyophilized material had a specific activity of 7 LD₅₀/ng corresponding to a loss of about 70% of the starting activity. Analysis of the lyophilized preparation by SDS-PAGE did not show that peptide bonds in the toxin molecule were hydrolyzed with formation of breakdown fragments.

The possibility of peptide bond hydrolysis was further examined using purified neurotoxin. The simplified system of purified type A neurotoxin showed that peptide bond hydrolysis was occurring during lyophilization (Figure 6). The extent to which the hydrolysis occurred in the soluble material and the insoluble material in the reconstituted lyophilized cake was markedly different. Lyophilized type A neurotoxin was analyzed on a linear 12.5% polyacrylamide Phastgel (Pharmacia). The soluble material containing 3-4 µg of toxin soluble in dH₂O showed a doublet in the region of the light chain. The doublet occurred to a much larger extent in the insoluble, aggregated material (Figure 6, lane 5). The difference in molecular weights of the two bands is about 2 kDa. When the aggregated material following lyophilization was run on a longer gel (ca. 12 cm, 10% polyacrylamide gel using the BioRad Protean II system), the reduced toxin showed the 50-52 kDa doublet and the unreduced toxin showed a doublet at about 143-145 kDa (Figure 7).

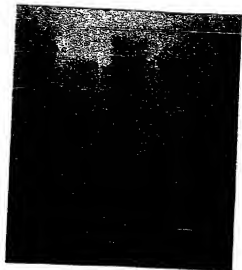


Figure 5. SDS-PAGE analysis of type A toxin complex before and after lyophilization. Lanes 1 (unreduced) and 2 (reduced with 0.5% w/v dithiothreitol), unlyophilized type A toxin complex, 4-5 μ g protein; lane 3, molecular weight markers (in kDa): rabbit myosin, 205, *E. coli* β -galactosidase, 118, rabbit phosphorylase b, 94, bovine serum albumin, 66, chicken egg albumin, 45, bovine erythrocyte carbonic anhydrase, 29, (Sigma), 5 μ g protein; lanes 4 (unreduced) and 5 (reduced), lyophilized type A toxin complex, 4-5 μ g protein.

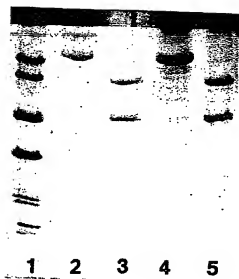


Figure 6. SDS-PAGE showing hydrolysis of purified type A neurotoxin during lyophilization. Lane 1, type A toxin complex (reduced with 0.5% dithiothreitol (w/v)), 4-5 μ g protein; lane 2, lyophilized, purified type A neurotoxin, water soluble fraction (145 kDa), 3-4 μ g protein; lane 3, lyophilized, purified type A neurotoxin, water soluble fraction (reduced) (93 kDa, 52 kDa), 3-4 μ g protein; lane 4, lyophilized, purified type A neurotoxin, water insoluble fraction (145 kDa), 4-5 μ g protein; lane 5- lyophilized, purified type A neurotoxin, water insoluble fraction (reduced), 4-5 μ g protein.

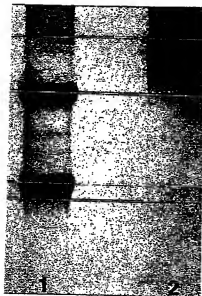


Figure 7. SDS-PAGE showing hydrolysis of light chain of type A neurotoxin during lyophilization. Lane 1, lyophilized, purified type A neurotoxin (reduced with 0.5% dithiothreitol (w/v)), 93 kDa, 52 kDa, 50 kDa, 7-10 μ g total; lane 2, lyophilized, purified type A neurotoxin, 145 kDa, 143 kDa, 4-5 μ g total.

The 50 and 52 kDa bands of the light chain doublet were separated on a 12 cm 12.5% SDS-PAGE gel and the individual bands excised and digested according to the method of Cleveland et al. (1977). The excised bands were digested with *Streptomyces griseus* pronase A and the fragments separated on a linear 15% polyacrylamide gel (Figure 8). The sample in lane 1 was digested with 16 ng of pronase, while lane 2 was undigested; both lanes showed the 52 kDa band. Lanes 3 (undigested) and 4 (digested with pronase) contained the second (50 kDa) band of the light chain doublet. The pattern of digestion fragments in lanes 1 and 4 are similar indicating homology between the 50 and 52 kDa bands. Lane 5 and 6 contained undigested 50 and 52 kDa bands while lane 7 contained 93 kDa heavy chain digested with pronase. Lane 8 contained 93 kDa heavy chain that was digested with 30 ng of trypsin while lane 9 contained the same 93 kDa heavy chain band digested with 15 ng of trypsin. The lack of homology between the digestion fragments in lane 7 and those in lanes 1 and 4 indicates that the origin of the 50-52 kDa doublet is from the light chain of the toxin molecule.

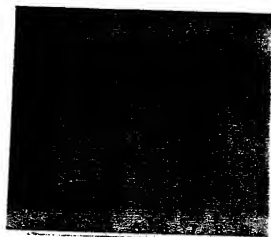


Figure 8. Partial proteolytic digestion of 52 kDa and 50 kDa light chain bands on 15% SDS-PAGE. Lane 1, 52 kDa type A neurotoxin light chain band excised from SDS-PAGE gel shown in Figure 7 plus 16 ng *Streptomyces griseus* pronase A; lane 2, undigested 52 kDa light chain band; lane 3, undigested 50 kDa light chain band; lane 4, 50 kDa type A neurotoxin light chain band excised from SDS-PAGE gel shown in Figure 7 plus 16 ng *Streptomyces griseus* pronase A; lane 5, undigested 50 kDa light chain band; lane 6, undigested 52 kDa light chain band; lane 7, 93 kDa heavy chain band from SDS-PAGE gel shown in Figure 7 plus 16 ng *S. griseus* pronase A; lane 8, 93 kDa heavy chain band from SDS-PAGE gel shown in Figure 7 plus 30 ng trypsin; lane 9, 93 kDa heavy chain band from SDS-PAGE gel shown in Figure 7 plus 15 ng trypsin.

Amino acid sequencing.

Amino acid sequencing was done on the 50 and 52 kDa bands to determine the residue at which hydrolysis was occurring. The bands were transferred to PVDF membrane by the procedure of Matsudaira (1987). The 52 kDa band gave a sequence of P-F-V-N-K-Q-F-N-Y-K-x-P-V-N-G-V-D- upon Edman degradation. During the sequencing run, the signal strength dropped to near the resolution limit of the instrument after the valine 13 residue. The sequence was still readable after this but at a lower limit of detection. The 50 kDa band gave strong P and V signals in the first two cycles of Edman degradation but the remainder of the sequence was N-terminal blocked.

Discussion

Studying the mechanisms of inactivation of therapeutic levels of *C. botulinum* toxin during lyophilization and storage is difficult due to the fact that nanogram quantities of toxin are in each vial. In order to study possible denaturing mechanisms such as aggregation, peptide bond hydrolysis, and deamidation much larger quantities of the toxin had to be examined. It was found in this study that a self-protection effect occurred in which the toxin acted as its own stabilizing excipient when microgram quantities of toxin were lyophilized. Lyophilization of therapeutic levels of type A toxin complex (20-1,000 LD₅₀) using certain formulations resulted in a much larger percentage of toxin inactivated (>90% inactivated) (Goodnough and Johnson, 1992) than was found in this study (20-32% inactivated) using microgram quantities. Therefore, the exact mechanisms by which therapeutic levels of toxin are inactivated on drying are extrapolated from the data obtained with much larger quantities of toxin. This has been shown to be different from drying lower levels of toxin.

Aggregation of proteins occurs when the conformation of the molecule changes to allow more hydrophobic regions buried in the center of molecule to become exposed to the aqueous solvent system or other hydrophobic areas thus favoring hydrophobic-hydrophobic interactions between individual protein molecules. Our results indicated that aggregation of toxin occurred during lyophilization. Toxin stored above its glass transition temperature (trial 1) could have allowed degradative chemical reactions such as deamidation and oxidation to occur. Storage in this condition may have allowed the samples to pick up water from the environment which was shown to cause further aggregation in other protein systems (Liu et al., 1991). The difference in recovery of material in trial 1, 2, and 3 may have been related to the smaller amount of material used in trial 1 giving a larger percentage of material which may have adhered to the vial. The difference in the specific activities

indicated that there could be additional inactivating events occurring during storage of the dried material. It is also possible that "self-protection" occurred in the trial with higher concentrations of toxin.

It is possible that there are oxygen dependent inactivation events occurring during lyophilization and storage of the dried toxin. The oxygen concentration in a partially frozen aqueous system at -3°C is 1,150 times higher than that at 0°C (Schwimmer, 1981). Cysteine residues have been shown to undergo auto-oxidation to form intra- or inter-molecular disulfide bonds as well as form oxidative degradation products such as sulfenic acid (Torchinsky, 1981). These oxidation reactions are greatly accelerated by the presence of metal ions such as copper and iron. No intra-molecular disulfide bond redistribution was seen in these studies as judged by non-reducing SDS-PAGE. However, it is possible that intermolecular disulfide bonds were rearranged causing inactivation of the toxin since such arrangements should not change the SDS-PAGE electrophoretic pattern.

Aggregation could be promoted by the dried material slowly picking up moisture from the environment. It has been shown with bovine serum albumin that moisture adsorbed by the solid, freeze-dried product caused aggregation in part due to thiol-disulfide interchange (Liu, et al., 1991). Other water dependent reactions such as deamidation and peptide bond hydrolysis would also be expected to be more prevalent in such cases.

Aggregation of the toxin complex is difficult to detect using standard size-exclusion chromatography as the native complex eluted near the exclusion limit. However, since there was no discernible broadening of the peak of lyophilized toxin it is possible that the conditions used (i.e. 100 mM sodium phosphate, pH 7.0) allowed dissociation of aggregates to the native MW of 800-900 kDa.

Deamidation appeared to be occurring in all cases. Unlyophilized type A and B neurotoxins as well as the lyophilized neurotoxins were substrates of the enzyme (Table 2). Type B neurotoxin seemed to be a better substrate than type A for the enzyme on a weight

basis. The assays were not done at low enough neurotoxin concentrations to quantitatively determine the number of isoaspartyl residues per toxin molecule. Low substrate concentrations are a requirement of the PIMT enzyme due to its extremely slow turnover rate (Johnson and Aswad, 1991). Further evidence for the existence of isoaspartyl residues came from amino acid sequencing of the hydrolyzed light chain. During sequencing of the 52 kDa light chain band, the signal was identical to the light chain of that of Binz et al. (1990) except for the absence of the first methionine residue. However, after the automated sequencer read through the first thirteen residues up to V13, the signal strength dropped substantially to a point just above background levels. The weaker sequence from valine-13 on to the wash out of the signal was identical to that published for type A light chain. Since pmol quantities of material were sequenced, it was not possible to quantitate the decrease in signal. The decrease in signal strength at valine-13 could be explained by a portion of the material being sequenced having some type of N-terminal blockage at the residue following valine-13. The next residues after valine-13 are the first asparagine-glycine sequence in the type A amino acid sequence. Since Edman degradation is stopped by isoaspartyl residues it is possible that deamidation could be occurring here. Such a decrease in signal strength could be used in future experiments to quantitate the proportion of deamidation at a given amino acid residue. The positions of the six asparagine-glycine sequences in type A neurotoxin as well as the eight asparagine-serine sequences are shown in Table 3. These sequences have been shown to be the most labile to deamidation under physiological conditions (Liu, 1992).

Table 3. Positions of asparagine-glycine and asparagine-serine amino acid sequences in type A neurotoxin (adapted from Goodnough and Johnson, 1994, in press).

| | <u>Light chain</u> | <u>Heavy chain</u> |
|---------|------------------------------------|--|
| Asn-Gly | -15,16- -178,179- -402, 403- | -539,540- -1032,1033- -1243, 1244- |
| Asn-Ser | | -570,571- -798,799- -935,936- -954,955- -971,972- -1026,1027- -1093,1094- -1151,1152- |

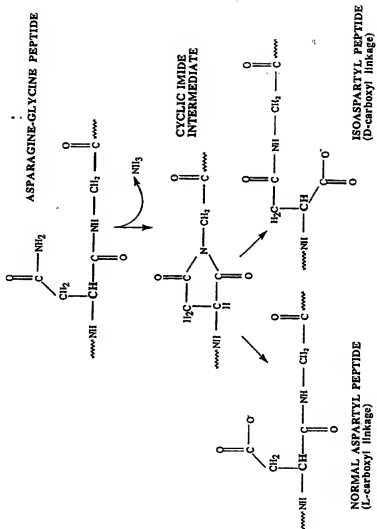
Deamidation requires regional flexibility in the peptide chain for the imide ring structure to form. The formation of the imide ring as well as two cleavage products of the ring structure are shown in Figure 9.

The regional flexibility of the peptide chain of type A botulinum toxin was examined using the method of Ragone et al. (1989) and a five amino acid floating window (D. Aswad, personal communication, 1993). The flexibility plot is shown in Figure 10. The first asparagine-glycine combination at N15G shows a relatively high degree of flexibility indicating that deamidation would be favored. The aspartate-glycine sequence at D141G in the light chain region appears to be very flexible as do the N971S, N1093S, and N1243G sequences of the heavy chain region. Additional research including peptide mapping will be necessary to determine to what degree these sequences are deamidated.

Further support for N-terminal blockage of the light chain sequence was obtained by examination of light chain fragmentation. The sequence determined from the lower molecular weight band of the light chain doublet (50 and 52 kDa) was completely N-terminally blocked after the third cycle of the sequencer. The first two cycles showed residues one and two to be proline and valine, respectively. Residue number three of the 50 kDa band would correspond to the asparagine-14 residue of the intact light chain. This is the first asparagine-glycine sequence in the light chain.

Isoelectric focusing (IEF) is one method of detecting deamidation as the pI shifts accordingly with the loss of amino groups from asparagine residues with the consequent gain of a carboxyl group. It is problematic to detect a change in pI by IEF with purified type A toxin as the molecule is too large to show a charge shift from losses of a minimal number of amino groups (Johnson, et al., 1989b; Liu, 1992). It may be possible in the future to show shifts in IEF of fragments of purified type A and B neurotoxin.

Figure 9. Mechanism of deamidation (adapted from Liu, 1992).



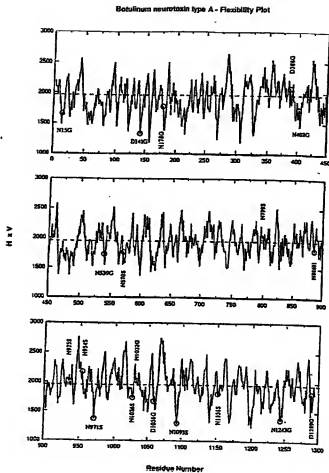


Figure 10. Flexibility plot of type A botulinum neurotoxin using a five amino acid floating window. Regions with higher flexibility have a lower $H \times V$ number.

Peptide bond hydrolysis was not observed during initial studies involving type A toxin complex. However, when purified toxin was substituted for the complex, hydrolysis was seen at the aspartate 11-proline 12 linkage as determined by amino acid sequencing. There are at least two reasons why hydrolysis of toxin in the complex was not detected. First, the samples of toxin complex that were electrophoresed after lyophilization and reconstitution were solubilized in dH_2O and the insoluble material separated by centrifugation. When neurotoxin was analyzed, the aggregated material was found to have a higher percentage of hydrolyzed peptides and may have been removed during sample preparation. The second reason hydrolysis was not detected in lyophilized toxin complex is that the migration rates of the fragmented toxin was nearly equivalent to that of two of the non-toxic proteins associated with the toxin in the complex. If the unreduced toxin present in the toxin complex was hydrolyzed at the same point as purified toxin, the lower molecular weight band of the doublet present at 143-145 kDa would run very close to the 118 kDa non-toxic complex protein which may have masked the detection. In the reduced sample, the fragmented light chain running at ca. 50 kDa would overlap the non-toxic complex protein running at about the same molecular weight.

Amino acid sequencing showed that the aspartate-proline bond at the N-terminus of the light chain was cleaved during lyophilization. It has been shown that sodium phosphate buffered systems can change pH during the freeze-concentration step of lyophilization. The pH of a sodium phosphate buffered solution at pH 7 can decrease to 5 during the freezing portion of a lyophilization cycle (Pikal, 1990; Van den Berg, 1966). Additionally, it has been shown that type A neurotoxin is susceptible to acid hydrolysis of the aspartate-proline bonds of the heavy chain at pH values as high as 5 (DasGupta and Tepp, 1991). The short 12 amino acid peptide resulting from cleavage of the N-terminal aspartate-proline bond has a molecular weight of about 1400 daltons. Such a molecular weight correlates well with the observed differences between the 50 and 52 kDa bands. The origin of the

fragment must be the N-terminus of the light chain since the partially hydrolysed, unreduced samples run with molecular weights of 143 and 145 kDa. If the fragment were being hydrolyzed from the C-terminus of the light chain, then the fragments would be running at 50 and 93 kDa without being reduced since the cysteine residue involved in the disulfide bridge connecting the two chains would have been excised. Also, if the fragment originated from the C-terminus of the heavy chain, the light-chain doublet would not be seen in reduced samples, instead the heavy chain would show a doublet upon reduction.

In conclusion, the mechanisms of inactivation of botulinum toxin are complex and varied. These mechanisms involve aggregation, deamidation, and peptide bond hydrolysis. Further study of the inactivation mechanisms will include the following; peptide mapping, isoelectric focusing of purified fragments of the toxin before and after lyophilization and comparison to measurements of isoaspartyl residues in the lyophilized and unlyophilized fragments. Such experiments will give a clearer picture of the events occurring during the freeze-drying process.

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CHAPTER VII

Immune response of rabbits to low doses of type A botulinum toxin.

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Abstract

Sublethal doses of various preparations of *Clostridium botulinum* type A toxin including Botox® and Dysport® were tested in a rabbit model for immunogenicity. Known quantities of various type A toxin preparations were injected over a period of time and the animals serum assayed for antibodies capable of neutralizing a small challenge of purified type A toxin in mice. Animals injected with a total of 18.3 and 18.1 ng of Botox® developed neutralizing antibodies to the toxin in 60 and 63 days. Animals injected with 5.89 ng of crystalline type A toxin complex from our laboratory produced antibodies that neutralized the challenge in 118 days. Animals injected with 0.92 ng of purified type A neurotoxin over 118 days did not produce neutralizing antibodies. Likewise, 5.89 ng of Dysport® administered over 165 days did not produce neutralizing antibodies.

Introduction

Clostridium botulinum is a diverse group of organisms capable of producing a potent proteinaceous neurotoxin. Different serotypes of C. botulinum produce neurotoxins that differ in primary amino acid sequence and thus are antigenically distinct. There are seven antigenically different serotypes currently recognized, A, B, C₁, D, E, F, and G. The organisms producing these toxins categorized by biochemical and growth requirements (Hatheway, 1990). The toxin causes a flaccid paralysis by binding to the pre-synaptic junction of motor neurons and inhibiting the release of the neurotransmitter acetylcholine (Schantz and Johnson, 1992; Simpson, 1981). The particular muscle or muscle group innervated by this particular motor neuron does not receive the signal to contract resulting in flaccid paralysis. Classically, this condition which is termed botulism has been linked to the consumption of food products which contained the preformed toxin. C. botulinum can also colonize wounds and the infant bowel causing a toxicoinfection (Sugiyama, 1980).

Since the early 1980's, investigations by Drs. Edward Schantz and Alan Scott have led to use of type A toxin for treatment of various spastic muscle disorders as an alternative to surgical techniques. The toxin is used in nanogram quantities and is injected directly into the affected muscle groups. Small enough quantities are used to ensure that the dose is effective but does not spread to other regions of the body. Originally, only a few indications were approved for treatment by the United States Food and Drug Administration including blepharospasm, hemifacial spasm, and strabismus. The use of the toxin to treat other focal dystonias such as spasmodic torticollis, writer's cramp, vocal dystonias, and club foot in children has led to ever increasing use for a variety of neurological disorders. Unfortunately, one of the major side-effects of the use of certain protein pharmaceuticals in high enough quantities is the production of antibodies by the patient to the drug. The presence of circulating antibodies has been demonstrated in

patients receiving large doses of the commercially available toxin (Jankovic and Schwartz, 1991).

We have developed a rabbit model in which repetitive injections of various type A toxin preparations have been given to simulate the treatment of a focal dystonia in order to assess the immunogenicity of the toxin. The model consists of injecting albino rabbits with sub-lethal doses of the toxin over a period of time and assaying the serum of the animals for the ability to neutralize a small but carefully quantitated amount of purified type A toxin. Our results show that the product presently available in the United States is the most antigenic of all the preparations tested to date while purified type A toxin and the product commercially available in Europe are the least antigenic. These results indicate that high specific activity preparations reduce the probability of patients developing neutralizing antibodies.

Materials and Methods

Animals

Female, ICR mice, 18-22 g (Harlan Sprague Dawley, Madison, WI) were used in toxin and serum titrations. New Zealand, albino rabbits, 4-6 lbs. were obtained from Hazelton Laboratories, Kalamazoo, MI.

Bacterial strains and growth conditions

The Hall A strain of type A *C. botulinum* was used to produce crystalline type A complex. This strain was originally obtained from Dr. J. H. Mueller at Harvard University and was further screened for high toxin titers at Fort Detrick, MD by Dr. E. J. Schantz and coworkers. This strain is routinely used for production of type A botulinum toxin due to high toxin titers and the rapid onset of cell lysis (usually within 48 h).

Stock cultures of *C. botulinum* Hall A were grown statically in 15 ml Hungate tubes containing 10 ml of cooked meat medium + 0.3 % dextrose (CMM, [Difco Laboratories, Detroit, MI]) under an anaerobic atmosphere (80% N₂, 10%CO₂, 10%H₂) at 37°C for 24 h and frozen at -20°C until use. CMM cultures of the Hall A strain gave toxin titers in excess of 10⁶ LD₅₀/ml within 48-72 h.

For toxin production, cultures of Hall A were grown statically in 15 liter volumes of toxin production medium (TPM) consisting of 2.0% NZ TT (lot # 9NC29) casein hydrolysate (Sheffield Laboratories, Norwich, NY), 1.0% yeast extract (Difco), and 0.5% dextrose, pH 7.3-7.4, for 5-7 days at 37°C. Cultures of Hall A showed heavy growth in this medium during the first 24-48 h followed by autolysis of the culture which was evident as a clearing and settling over the next 48-120 h.

Type A toxin complex.

Type A toxin complex was purified according to a modification the method of Duff et al. (1957). Briefly, the method involves a series of precipitations and extractions using low pH, ethanol, and crystallizations of the toxin complex for purification from crude culture (Schantz, 1964).

Commercially available type A toxin complex preparations (Botox® and Dysport®) as well as a new type A toxin product currently undergoing clinical trials (ASB) were obtained from Dr. Gary Borodic, Massachusetts Eye and Ear Infirmary, Boston, MA.

Type A neurotoxin purification.

Type A neurotoxin was purified from the associated non-toxic proteins of the complex by a modification of the method of Tse et al. (1982). Toxin complex in 50 mM sodium citrate buffer was eluted from a 1 liter DEAE-Sephadex A50 column (5 cm x 65 cm) and precipitated by addition of 39 g of solid ammonium sulfate/100ml. The precipitated toxin complex was collected by centrifugation (12,000 x g, 5-10°C, 20 min), dialyzed against 25 mM sodium phosphate, pH 7.9, and applied to a DEAE-Sephadex A50 column equilibrated with the same buffer. The binding capacity of this particular matrix under these conditions is 0.9 mg of complex/ml of swollen gel. Various size columns were utilized by applying ca. 90% of the column binding capacity. Toxin was separated from the non-toxic proteins of the complex and eluted from the column with a linear 0-0.5M sodium chloride gradient. Toxin eluted from the column in the first protein peak and fractions which had 260/278 nm absorbance ratios <0.6 were pooled and precipitated by adding 39 g of solid ammonium sulfate/100 ml. Material recovered from the DEAE-Sephadex A50 column at pH 7.9 was further purified by chromatography on SP-Sephadex C50. Precipitated toxin from DEAE-Sephadex A50 columns at pH 7.9 was collected by centrifugation (12,000 x g, 5-10°C, 20 min) and dialyzed against 25 mM sodium

phosphate, pH 7.0. The dialyzed toxin was applied to 25 ml SP-Sephadex C50 in 25 mM sodium phosphate, pH 7.0. Contaminating material did not bind to the column under these conditions. The toxin was eluted with a linear 0-0.25 M sodium chloride gradient.

SDS-gel electrophoresis.

Electrophoresis was performed using a Pharmacia Phast System (Pharmacia LKB Biotechnology, Piscataway, NY) and 12.5% linear pre-cast gels according to the manufacturers instructions. Sample buffer consisted of 75 mM Tris-HCl (Sigma Chemical Co., St. Louis, MO), 5 M urea (Sigma), 5% SDS (Sigma), and 20% glycerol (Sigma), pH 6.8. All samples were boiled for 5-10 min. Some samples were reduced by the addition of 0.5% (w/v) dithiothreitol. Bands were visualized by staining in 0.1% Coomassie brilliant blue R250 in 5:5:2 dH₂O:methanol:acetic acid, destaining by diffusion in 9:3:3:1 dH₂O:methanol:acetic acid. Some gels were silver stained according to the procedure of Hames and Rickwood (1990).

Titration of toxin samples.

Toxin sample titers were estimated using the intravenous method of Boroff and Fleck (1966) in groups of 5 mice per dilution. Titers were determined more accurately using the standardized intraperitoneal method of Schantz and Kautter (1978) with 5 mice per dilution.

Toxin standard.

A toxin standard was prepared using purified type A neurotoxin in 50mM sodium acetate, 2 mg/ml gelatin (Difco), 3 mg/ml bovine serum albumin (Sigma), pH 4.2, according to the method Schantz and Kautter (1978). The standard was stored at 4°C. The toxin standard was titrated according to the method of Schantz and Kautter (1978) using

seven mice per dilution. The standard contained 56 LD₅₀/ml when freshly prepared. When titrated 9 months later in the same fashion, the standard contained 60 LD₅₀/ml which was within the $\pm 15\%$ margin of error given by Schantz and Kautter (1978) for intraperitoneal bioassay of botulinum toxin.

Sub-lethal injection of rabbits.

Rabbits were injected on the days indicated in Table 1. Initially, 0.1 ml injections were made intramuscularly in the hind legs with subsequent 0.1 ml boosts given subcutaneously over the front shoulders. A total of 0.2 ml was given to each animal on each day indicated which amounted to 12-15 LD₅₀/boost. Blood samples were drawn from the central vein in the ear. Serum samples were taken on the same days just prior to injection. Five ml samples of whole blood were incubated on ice for 1 h at which time the serum was separated by centrifugation at 5,000 rpm in a Sorval SS-34 rotor at 4-10°C for 20 min. Samples were kept frozen at -20°C until assayed for type A toxin antibodies.

Enzyme-linked immunosorbent assay (ELISA) of toxin samples.

A modified ELISA was used for determination of the number of nanograms of toxin/ml of the reconstituted commercial products. Sandwich complexes consisting of the toxin preparation being tested, chicken immunoglobulin Y specific for type A toxin, and horse immunoglobulin G specific for type A toxin conjugated to Russell's viper venom factor XA (RVV-XA) activating enzyme were formed in solution. The complexes, consisting of the two different antibodies bound to the toxin, were captured on a microtiter plate coated with rabbit immunoglobulin G specific for chicken immunoglobulin Y. After capture, a mixture of coagulation factors II, V, and X were added. A positive result indicating the presence of toxin generated thrombin due to the presence of RVV-XA. Alkaline phosphatase labelled fibrinogen was added along with polystyrene pegs coated

with fibrinogen. Thrombin caused hydrolysis of fibrinogen to fibrin which resulted in deposition of labelled fibrin onto the polystyrene pegs. The pegs were then removed, washed, and placed in phenolphthalein monophosphate as a substrate for alkaline phosphatase (Doelgast, et al., 1993). The assays were performed by Dr. Mike Roman, Kraft General Foods, Glenview, IL.

Results

Purification of type A toxin complex.

The batch of toxin complex used in these experiments was recrystallized three times and had a specific toxicity of 18 mouse intraperitoneal 50% lethal doses/ng (LD₅₀). The absorbance ratio at 260/278 nm which is used as one measure of purity (Schantz and Johnson, 1992) was 0.52 indicating that the preparation was relatively free of contaminating nucleic acids. SDS-gel electrophoresis demonstrated bands (from top of gel down in kDa) of 145, 118, 48, 35, 29, 22, 21, and 15 which are indicative of type A complex (Figure 1, lane 4) (Johnson and Goodnough, 1993, in press). When the sample was reduced with dithiothreitol the two bands indicative of type A toxin heavy chain (93 kDa) and light chain (52 kDa) were observed (Figure 1, lane 5). For injection into rabbits the type A complex was diluted to 60 LD₅₀/ml in 30mM sodium phosphate, 0.2% gelatin, pH 6.4 (gel-phosphate).

Purification of type A neurotoxin.

Type A neurotoxin was purified to homogeneity (Figure 1, lanes 2 (unreduced) and 3 (reduced)). The 260/278nm absorbance ratio of the preparation was 0.50. The specific toxicity was approximately 96 LD₅₀/ng. For injection into rabbits the type A neurotoxin was diluted to 60 LD₅₀/ml in gel-phosphate, pH 6.4.

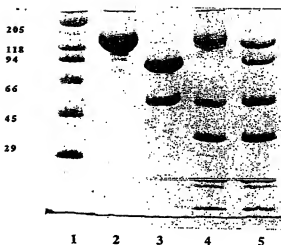


Figure 1. SDS-PAGE analysis of type A toxin preparations. Lane 1, molecular weight markers (in kDa); rabbit myosin, 205, *E. coli* β -galactosidase, 118, *E. coli* phosphorylase, 94, bovine serum albumin, 66, ovalbumin, 45, carbonic anhydrase, 29, (Sigma Chemical Co., St. Louis, MO), 5-6 μ g protein total; lane 2, purified type A neurotoxin, 4-5 μ g protein; lane 3, purified type A neurotoxin (reduced with 0.5% (w/v) dithiothreitol), 4-5 μ g protein; lane 4, type A toxin complex, 4-5 μ g protein; lane 5, type A toxin complex (reduced), 4-5 μ g protein.

Titration of Botox[®], Dysport[®], and ASB.

Botox[®], the commercial type A toxin product of Allergan, Dysport[®], the commercial product of Porton Down, and ASB were titrated using the method of Schantz and Kautter (1978) with five mice per dilution. Two vials of Botox[®] had 70 LD₅₀/ml and 66 LD₅₀/ml when reconstituted in 2.0 ml of dH₂O. One vial of Dysport[®] had a titer of 217 LD₅₀/ml when dissolved in a total of 2.0 ml of dH₂O. This material was diluted to 60 LD₅₀/ml in gel-phosphate, pH 6.4, prior to injection in rabbits. Two vials of ASB contained 69 and 72 LD₅₀/ml when treated in a similar fashion.

Serum titrations.

Rabbit serum was assayed for neutralizing antibodies by combining 0.1 ml of the toxin standard along with 0.5 ml of a serum dilution and 0.6 ml of gel-phosphate, pH 6.4. The solution was incubated at room temperature for 30-60 min with occasional mixing by inversion. One-half ml of the solution was injected intraperitoneally into each of two mice. Mice were observed for signs of botulism for a period of four days. Serum samples which neutralized type A toxin were diluted in two-fold increments and the neutralization assay repeated in the same fashion to determine the neutralizing titer of the serum.

Serum titrations.

Pre-immune serum samples were all negative for antibodies capable of neutralizing type A toxin (Table 1). Antibodies were not detected up to day 118 for the animal given type A toxin complex and day 60 and 63 for the Botox[®] animals. Titers of type A antibodies continued to rise in the second Botox[®] animal (Botox[®] II) with repeated boosts of the toxin. Numbers in brackets following a day indicated the highest two-fold dilution which neutralized 5.6 LD₅₀ of the type A toxin standard.

Table 1. Immune response of rabbits to sub-lethal doses of type A botulinum toxin.

| Toxin sample | Days of injection/titration ^a | Total ng given | Specific toxicity ^b |
|----------------------|--|----------------|--------------------------------|
| A neurotoxin | 0, 29, 42, 56, 69, 88, 107, 118 (no antibodies detected) | 0.92 | 96 |
| A complex | 0, 29, 42, 56, 69, 88, 109, 118 (1:2) | 5.12 | 18 |
| Botox [®] | | | |
| I* | 0, 21, 35, 49, 60 (1:1) | 18.3 | 4.3 |
| II* | 0, 21, 35, 49, 63 (1:1), 77 (1:2), 84 (1:4) | 25.4 | 4.3 |
| Dysport [®] | 0, 21, 32, 46, 60, 67, 81, 95, 109, 123, 137, 151, 165 (no antibodies detected) | 5.89 | 25.6 |
| ASB | 0, 21, 35, 49, 60 (no antibodies detected) | 4.08 | 17.3 |

*Two separate animal trials designated I and II are represented.

^aAll antibody samples were titrated against 5.6 LD₅₀ of purified type A neurotoxin according to the following: 0.5ml serum + 0.1ml containing 5.6 LD₅₀ type A toxin + 0.6ml gel-phosphate, pH 6.4. The solution was incubated at room temperature for 30-60 minutes. Two mice per two fold dilution were injected intraperitoneally with 0.5ml of serum + toxin mixture. Dilutions which neutralized the toxin challenge are indicated in brackets. ^bLD₅₀/ng.

ELISA results.

The ELISAs performed on Botox[®], Dysport[®], and ASB which when corrected for the known concentration of ASB (8.15 ng/vial) gave values of 36.3 ng/vial of Botox[®] and 16.95 ng/vial of Dysport[®]. It should be noted that both ASB and Botox[®] are labelled to contain 100 LD₅₀/vial while Dysport[®] is labelled to contain 500 LD₅₀/vial. These results indicated that Botox[®] had an average specific toxicity of 4.3 LD₅₀/ng, Dysport[®] had 25.6 LD₅₀/ng, and ASB had an average specific toxicity of 17.3 LD₅₀/ng after reconstitution.

Discussion

The likelihood of antibody formation in patients treated with Botox® seems to depend on the amount of material used to treat the particular disorder as well as the number of treatments received by each patient (Jankovic and Schwartz, 1991; Borodic et al., 1991). Patients who receive higher doses of toxin on a more frequent basis are those who are more likely to produce neutralizing antibodies to the toxin. In particular, those patients with spasmodic torticollis are at high risk due to the large doses of toxin which are spread over a large area. We have used a rabbit model for testing the immunogenicity of various toxin preparations in a manner similar to their intended use, i.e. by injection of sub-lethal doses on a repetitive basis. In this study, neutralization of a small but accurately quantitated amount of toxin gave greater sensitivity in detecting antibodies than previously reported (Hatheway et al., 1984). In our assay, as little as 0.0012 IU/ml of type A specific antibodies could be measured.

We have compared the two currently available commercial products (Botox® and Dysport®) to two type A toxin preparations made at the Food Research Institute and one type A toxin preparation currently undergoing clinical trials (ASB). The two preparations made at the Food Research Institute had specific activities that were 18 LD₅₀/ng for type A toxin complex and 96 LD₅₀/ng for purified type A neurotoxin. Botox® has been reported to contain 2.5 LD₅₀/ng which gives it the lowest specific activity among those tested. Our results of 4.3 LD₅₀/ng were slightly higher than this value. The data from the amplified ELISA assay correlates well with this estimate as $2.5 \text{ LD}_{50}/\text{ng} \times 36.3 \text{ ng} = 90.75 \text{ LD}_{50}/\text{vial}$. However, the average of the toxin titrations of the individual vials gives a specific toxicity of 3.7 LD₅₀/ng. Using the same approach, the specific toxicity of Dysport® used in this study was estimated to have a specific activity of 434 LD₅₀/vial / 16.95 ng/vial which equals 25.6 LD₅₀/ng. This is close to the specific activity expected

with chromatographically purified type A toxin complex that has been lyophilized under conditions allowing recovery of 75-85% of the active toxin (Goodnough and Johnson, manuscript in preparation).

Lesser quantities by weight of the preparations with higher specific activity were injected (see Table 1). On a weight basis, 10 LD₅₀ of purified type A neurotoxin with a specific toxicity of 96 LD₅₀/ng was equal to 104 pg. Ten LD₅₀ of the type A complex with a specific toxicity of 18 LD₅₀/ng which was purified at the Food Research Institute was approximately 550 pg. Since less toxin is being given, the immune system of the animal is exposed to less of the antigenic substance. Additionally, it has been shown that the non-toxic components of the complex including those with hemagglutinating properties are more antigenic than the toxin itself (Sakaguchi et al., 1974; George Doellgast, personal communication). Hence, the non-toxic components could be acting as adjuvants stimulating a stronger immune response than a more purified toxin. An additional adjuvant present in all vials of lyophilized toxin is toxoid of the specific toxin formed during handling and lyophilization of the toxin itself. This problem can be reduced by up to 10 fold by utilizing the appropriate formulation and drying conditions (Goodnough and Johnson, 1992).

Possible solutions to the problem of antibody formation in patients being treated for spastic muscle disorders include: a). use of botulin toxins with higher specific activities, including those which have been chromatographically purified from the more antigenic non-toxic and hemagglutinating fractions, and b). implementation of drying processes which result in a higher percent recovery of active toxin to minimize the formation and presence of toxoids of the toxin. Narrowing the range of permitted LD₅₀'s per vial by the Food and Drug Administration from $\pm 30\%$ (which allows for up to 130 LD₅₀ of toxin in a given vial) to some lower value (e.g. 10-20%) would reduce the chance of inadvertent administration of excess toxin. Such an excess could not only further stimulate the

production of antibodies by a patient but could also result in dangerous side-effects such as ptosis and inadvertant paralysis of muscles at sites distant from the injection.

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CHAPTER VIII

Conclusions and Future Prospects

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Conclusions and commentary.

There is a need for standardization regarding botulinum toxins as used in the medical field. This became very evident during the Interagency Botulinum Research Council Committee conference on botulinum toxins held in Madison, WI in December of 1993. The current definition of a unit of botulinum toxin used pharmaceutically is one unit of toxic activity is equivalent to 1 mouse intraperitoneal 50% lethal dose or 1 LD₅₀. There are a couple of difficulties, however, in using the term 'unit' when speaking of botulinum toxin. It has become apparent that some researchers and clinicians who use the toxin on humans do not have a full understanding of this convention. Frequently, the unit of toxin is written about and spoken of as an international unit. An international unit is a description of a quantity of anti-toxin not toxin. One international unit of antitoxin is equivalent to 10,000 LD₅₀ of types A, B, C, D, and F toxin. Also, one international unit of antitoxin is equivalent to 1,000 LD₅₀ of type E toxin. A standard unit needs to be defined for biological toxicity of botulinum toxin including the ng of toxin and non-neurotoxin proteins in each preparation.

An additional point of confusion is the definition of a 50% lethal dose of botulinum toxin. The dose is usually referenced in terms of the amount needed to kill half of a population of white mice. However, the goal in treating patients with botulinum toxin is not to kill half of the population but rather to treat a given muscle group with enough toxin to elicit the desired response. Another biological unit has been proposed, the median paralysis unit, which is less than an LD₅₀ but more difficult to quantitate as it currently involves injecting an amount of toxin into hind limb muscles of mice and rats and observing the regional paralysis and duration of the paralysis. More work will need to be done on this idea if it is going to be generally accepted and used consistently by independent laboratories. It will be necessary to develop a simplified procedure with a definite end point if it is going to supersede the LD₅₀ as a unit of measurement. An

additional complication is the fact that the various toxin serotypes have different durations of action. There is even evidence that different strains within a given serotype have differing durations of action. So, for a given amount of denervation needed to achieve relief from a particular dystonia, the amount of toxin used will be different for each serotype used and possibly for each strain in an individual serotype. Only serotype A produced by one strain is currently being used in the United States. It will be necessary to develop other serotypes and strains since an increasing number of patients are developing neutralizing antibodies to the current product.

There is a need for an alternative botulinum toxin product for treating spastic muscle disorders in the United States. The current product has a very low specific activity for type A toxin complex which when coupled with the present formulation used in the drying process (inclusion of sodium chloride and alkaline pH) results in a product with less active toxin than inactive toxoid. The consequences of this are that the patients being treated with the material are developing antibodies at an alarming rate. A different product that was designed to alleviate the above concerns would certainly be an improvement. The use of purified neurotoxins has been shown here to be possible in a pharmaceutical product. The use of such material in concert with a drying excipient that would allow the shipment and storage of the material at room temperature would reduce the major drawbacks with extended use of botulinum toxin as a therapeutic.

Future considerations that may spring from some of this research include some of the following: 1. new methods of determining the antigenicity of individual protein pharmaceutical preparations using *in vivo* methodology, 2. further investigation into the mechanisms of inactivation of protein pharmaceuticals during preparation, handling, and storage, and 3. development of additional botulinum toxin serotypes and combinations of serotypes to treat patients with focal dystonias.

The notion that the toxin and non-toxic binding proteins themselves are capable of passing through the intestinal barrier may be a novel method of delivery of other pharmaceutical compounds. This possibility was not explored in this thesis but may warrant further attention. Also, limited work has been done regarding the development of chimeric toxin molecules that exploit the specificity and binding affinity of the heavy chain of botulinum toxin. It may be possible to develop other pharmaceuticals that are targeted to the terminal neurons using this specificity.

Drug delivery is one aspect of botulinum toxin therapy that has not been seriously addressed. Possible future work could include the development of a sustained release formulation that would be similar to the contraceptive Norplant where the pharmaceutical is released slowly through diffusion from an inert carrier such as latex. Hypothetically named "Toxplant", the advantages of using such a delivery would be that the treatment intervals could be extended to many months or even years. The current treatments involve intramuscular injections every 2-3 months which can be painful. Further, very low amounts of the toxin would diffuse out from such a delivery vehicle maintaining the desired effect on the neuro-muscular junction while avoiding stimulation of the immune system by only releasing very small quantities at any given time.

Since there are different target proteins for each toxin serotype, combining two or more toxin serotypes may allow the use of much lower quantities of toxin through synergistic effects. In the same vein, the use of a combination of botulinum neurotoxin and a chimeric toxin that uses the binding specificity and affinity of botulinum neurotoxin heavy chain and the active portion of a toxin that would slow or inhibit the resprouting of nerve terminals (the primary reason for retreating patients) would be advantageous. Such a chimeric toxin might be one that inhibits protein synthesis such as some of the ADP-ribosylating toxins including diphtheria toxin, ricin, and botulinum toxins C₂ and C₃.